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ACTIONS OF SODIUM AZIDE

BY

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(Received July 23, 1948)

Hydrazoic acid or azoimide is a colourless liquid with explosive properties which solidifies at −80° C. and boils at 37° C. Azoimide gas has a characteristic pungent odour. First prepared by Curtius (1890) it is a triazo compound in which a complex of three nitrogen atoms acts as a monad radical. Hendricks and Pauling (1925) showed that the N_3 ion is constituted $N \rightleftharpoons N \rightleftharpoons N$ in chain form. Hydrazoic acid is a feebly dissociating acid. Smith and Wolf (1904) showed that it was toxic on inhalation by small animals, causing a fall in blood pressure, tachycardia, and stimulation of respiration, and that in solution it paralysed preparations of isolated frog muscle. Biehler (1927) observed that the gas was a convulsant in frogs, and Hildebrandt and Schmidt (1937) extended the observation to cats and also noted a stimulant effect on gut. Stern (1927) and Kocher (1930) described acute collapse in human beings from inhalation of the gas, and Graham, Robertson, and Rogan (1948) studied symptoms of hypotension in a group of workmen exposed to the fumes of azoimide intermittently over a period of years. Hydrazoic acid forms explosive salts with heavy metals, the lead salt being used as a detonator. Fairhall, Jenrette, Jones, and Pritchard (1943) have discussed the hazards of this substance as an industrial poison.

The azoimide salt of sodium is stable, neutral, and fully dissociated in solution (West, 1900). Loew (1891) published an account of experiments on the toxicity of sodium azide, in which he showed that approximately 40 mg./kg. was lethal to mice, while 30 mg. killed a dog in 104 min. from respiratory failure and cramp. Smith and Wolf (1904) showed that sodium azide had the same effect as azoimide in lowering blood pressure, stimulating respiration, and increasing the rate of the heart. They found that the isolated perfused heart of the rabbit was inhibited by a concentration of 1 in 90,000. Further observations have been reported by Graham (1948).

Most recent work with the azide radical has been confined to the investigation of the effect of sodium azide on cellular respiration. Keilin (1936), Keilin and Hartree (1934 and 1935), and Stannard (1939) have shown that azide interferes with cellular metabolism by inhibiting the oxidation processes in which cytochrome plays a part; it also inhibits indophenol oxidase and liver catalase, but not glycolysis. The salt is fungistatic and forms a compound with methaemoglobin *in vitro* but not *in vivo*.

Toxicity

The LD50 in groups of white mice was found to be 28–34 mg./kg. by intraperitoneal injection, 19 mg./kg. intravenously, and 27 mg./kg. by mouth. The LD5 i.p. was 23.7 mg./kg. The LD50 of sodium nitrite was 168 mg./kg. and of potassium thiocyanate 600 mg./kg. measured at the same time. According to Fairhall *et al.* (1943) the LD75 of NaN_3 for rats is 75 mg./kg. i.p.

Mice, rats, guinea-pigs, and rabbits injected with sodium azide by the oral, subcutaneous, intramuscular, intraperitoneal, or intravenous routes all showed similar symptoms, which varied only in degree and rapidity of onset with varying dosage levels of the salt. Sublethal doses caused a preliminary stimulation of respiration and might give rise to clonic convulsions. This phase was accompanied by increased urination and passage of faeces and was followed by a prolonged period of collapse such as is seen with nitrite and thiocyanate. Death occurred quietly with azide if the dose was such as to delay the termination for some hours, but if the dose was overwhelming death occurred acutely in convulsions. In these circumstances mice and rats assumed a character-

istic death posture with the head flexed, the forelimbs flexed and pronated, and the tail and hind limbs extended.

Cardiovascular System

Systemic blood pressure.—Small doses of sodium azide caused a sharp transient fall in arterial blood pressure in cats and rabbits, the former being more sensitive than the latter. The amount of this fall depended on the initial level of the blood pressure, the anaesthetic used, and the dose of azide given. Cats were most sensitive when anaesthetized with ether and given aqueous hydrazoic acid intravenously: under these conditions a fall of blood pressure of about 60 mm. Hg was observed with 0.5–1.0 μg. HN_3 per kg. In rabbits under urethane 5–100 μg. HN_3/kg. was needed to produce a similar effect.

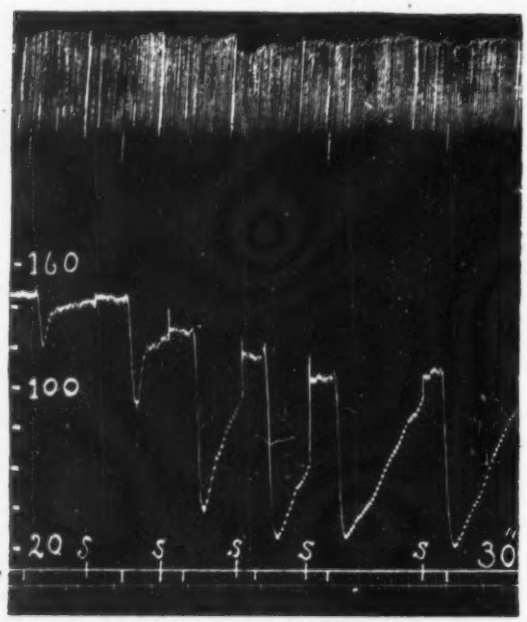

FIG. 1.—Cat ♂ 3.4 kg. Chloralose, 80 mg./kg. i.v. Upper line respiration (stethograph lever), next line carotid blood pressure, injection signal and drum stop (S), base line for zero blood pressure and time in 30 sec. Transient fall in blood pressure and stimulation of respiration occurs on each injection of 2 μg. NaN_3 at intervals of 5 min. The effect on respiration is minimal: the effect on blood pressure increases on repetition.

If the dose of sodium azide given intravenously to cats was small (1.0 μg./kg. or less) the response increased with repetition until it became constant, and the pressure did not return to its former level between doses (see Fig. 1). If the initial dose was larger (or in rabbits) this early increase in response with repetition of doses was not seen.

There was no diminution of response to repeated injections of sodium azide (5–15 μg./kg.) so long as the blood pressure was permitted to return to its initial level between doses. If the time interval between administrations of the azide was shortened or the dose given at the same intervals of time was increased, the blood pressure did not return to its original level. In such circumstances the effects of repeated doses of azide decreased and might be extinguished. As soon as time was given for restoration of the pressure approximately to its initial level the full effect of the original dose of azide was restored. This relationship between the initial level of the blood pressure and the response is illustrated in Fig. 2.

The fall in blood pressure resulting from administration of small doses of azide to cats or rabbits probably results from a direct action on the smooth muscle of the blood vessels. It occurred in anaesthetized eviscerated animals after atropine and vagotomy, after benadryl, and after excision of the carotid sinuses and adrenalectomy, and in spinal cats similarly prepared. The effect was not modified by previous injection of eserine or nicotine. Records of the volume of the spleen and foreleg showed a decrease in the volume of both accompanying the fall in pressure, but in three preparations out of ten the spleen dilated actively. Perfusion of the hind limbs of the rat through the aorta with a saline solution

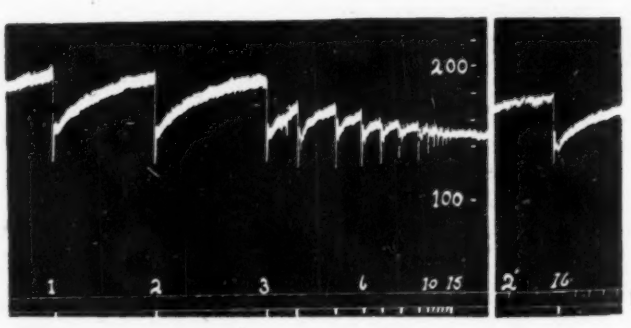

FIG. 2.—Cat ♂ 3.2 kg. Chloralose, 80 mg./kg. i.v. Upper line carotid blood pressure, middle line time in 2 min., lower line zero blood pressure and injection signal. 50 μg. NaN_3 in 0.25 ml. water was injected quickly at each signal. Nos. 1–3 were given at 15 min. intervals, nos. 4–6 at 5 min. intervals, nos. 7–10 at 2 min. intervals, nos. 11–15 at lesser intervals. Between nos. 15 and 16 an interval of 45 min. The degree of response to azide is related to the initial level of blood pressure.

under constant pressure and recording of the volume of the venous effluent showed that azide increased the rate of flow as a result of vasodilatation.

If the dose of sodium azide injected into cats was increased to 10–20 mg./kg. a rise in blood pressure occurred in the majority of animals, though some reacted with a severe and prolonged fall in pressure. The rise in blood pressure usually obtained was not modified by excision of the carotid sinus area or vagotomy. It was abolished by previous excision of the adrenal glands or by blocking agents such as dibenamine and dihydroergotamine. That the stimulation of the adrenals is an indirect one was proved by failure to repeat the effect after bilateral splanchnic nerve section and by the failure to obtain

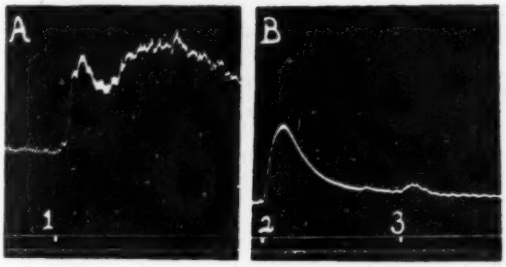

FIG. 3.—Cat ♂ 3.2 kg. Ether, chloralose 80 mg./kg. Upper line carotid blood pressure, middle line zero blood pressure and injections, lower line time in 10 sec. Preceding the records shown the cat received 10 mg. NaN_3/kg. i.v. (with effect as in A), and bilateral excision of the carotid sinuses. At 1 repeat of 10 mg. NaN_3/kg. giving prolonged rise in blood pressure. Between A and B bilateral splanchnic nerve section. At 2 adrenaline (2 μg./kg.). At 3 repeat 10 mg. NaN_3/kg. with no effect.

the effect in spinal cats (see Fig. 3). A similar rise in pressure was observed in rabbits after similar doses of azide.

The heart.—Injection of 50 μg.–1.0 mg. sodium azide into the inflow of the perfused heart of the cat (Langendorff preparation) caused an increase in the force but not in the rate of contraction: 2–5 mg. caused transient inhibition of some preparations. In the isolated rabbit heart the increase in force was less marked and inhibition was more often seen with the higher doses. In both species dilatation of the coronary vessels with increase in coronary flow occurred. Fig. 4 shows the effect of a dose of 50 μg. sodium azide on the perfused cat heart and the coronary flow from it. In view of the hypotensive effect of sodium azide on systemic blood pressure the action of other agents with a similar hypotensive activity was tested on the coronary circulation. The dilator effect of an equimolar amount of sodium nitrite was less intense but longer in duration than that of 50 μg. sodium azide; potassium thiocyanate had no effect (see Fig. 5).

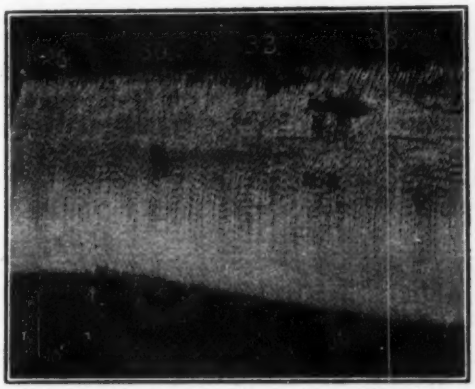

FIG. 4.—Record of the action of a perfused cat heart (Langendorff preparation). Time in 10 sec. The figures at the top are a measure of the coronary flow in ml./min. At the arrow 50 μg. NaN_3 was injected into the perfusing cannula.

The effect of intravenous injection of sodium azide on the heart of anaesthetized cats and rabbits was variable. In rabbits anaesthetized with urethane 10–100 μg. of azide increased the force and sometimes the rate of contraction of the heart, but in the majority of animals the heart was temporarily slowed. Atropine or vagotomy abolished this slowing but left the increase in force

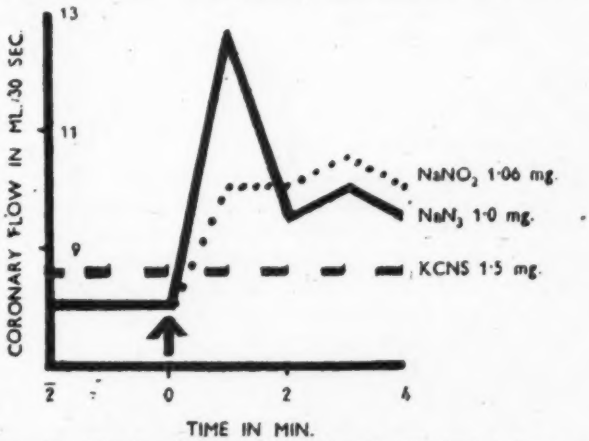

FIG. 5.—Graph of the coronary flow in the isolated perfused heart of the cat (Langendorff preparation). At intervals of 20 min. equimolar amounts of potassium thiocyanate, sodium nitrite, and sodium azide were injected (arrow). KCNS had no effect (interrupted line), NaN_3 had a powerful but short-lasting dilator effect (solid line), and $NaNO_2$ had a longer-lasting effect (dotted line).

unaffected. Larger doses of azide (5–10 mg. of azide/kg.) produced a marked initial slowing and inhibition of the heart followed by increase in cardiac activity above the initial level. This slowing was abolished by vagotomy or atropine, leaving the stimulation unaffected. In cats the effects of azide were similar, but this animal was more sensitive to sodium azide than the rabbit. A few μg. of azide per kg. brought about a marked increase in the rate and force of the heart (see Fig. 6): larger doses (5 mg./kg.) caused a transient inhibition, abolished by atropine or vagotomy and unobtainable in the spinal cat, followed by a prolonged increase in the rate and force of the heart,

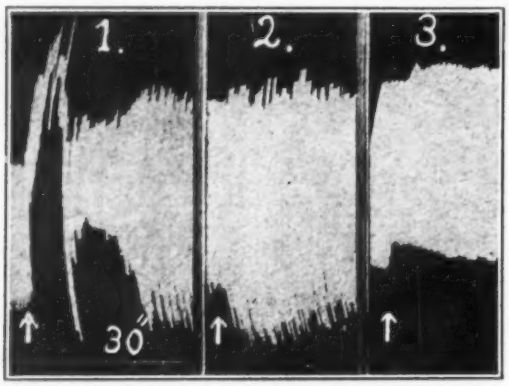

FIG. 6.—Direct myocardiograph tracing from cat ♂ 3.2 kg., ether and 80 mg. chloralose/kg. 1. Sodium azide (5 mg./kg.): transient inhibition followed by stimulation. 2. Repeat after bilateral cervical vagotomy and 1 mg. atropine sulphate/kg., followed by 15 mg. dibenamine HCl/kg.: stimulation without preliminary inhibition. 3. Repeat after bilateral adrenalectomy: absence of stimulation.

not affected by dibenamine (15 mg./kg.) but prevented by previous adrenalectomy (see Fig. 6). In a certain number of these animals, as already mentioned in the section dealing with the effects of azide on the blood pressure, the effect of large doses of the salt were mostly inhibitory with a fall in pressure replacing the rise in pressure. In these circumstances the cardiac action was also inhibited for a long period after injection, and adrenalectomy made little or no difference to the reactions obtained. Excision of the carotid sinus area did not modify these responses.

Since sodium azide in small doses appeared to have a more powerful effect than the other commonly used hypotensive agents on the blood flow in the systemic and coronary circulations comparison was made of their activities on the heart of the whole animal under light anaesthesia (ether, chloralose, or urethane). As is shown in Fig. 7 the

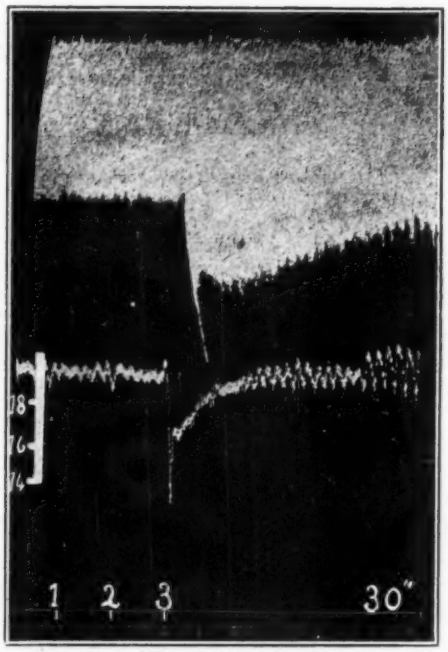

FIG. 7.—Cat ♂ 4 kg., 80 mg. chloralose/kg. i.v. Upper line direct myocardiograph lever, next line carotid blood pressure, injection signal, time in 30 sec. At 1, 2, and 3 injection of equimolar amounts of KCNS, $NaNO_2$, and NaN_3 (50 μg.) respectively. Note transient inhibition of heart followed by augmentation of the beat and abrupt fall in blood pressure after injection of azide.

characteristic cardiovascular action of azide—stimulation of the heart and fall in blood pressure—is obtained with amounts of azide such that equimolar amounts of nitrite and thiocyanate have no obvious effect.

Respiration

The rate and depth of breathing were increased by inhaled hydrazoic acid gas in very low concentrations (see Graham, Robertson, and Rogan, 1948, Fig. 1) and by injected azide in all doses. With doses of less than 1 μg. sodium azide per kg. the stimulant effect on respiration did not last so long as the accompanying fall in blood pressure. The degree of stimulation might be such as to cause marked irregularity of breathing and even generalized clonic convulsions. With suitable doses these convulsions appeared immediately after injection of the azide and were not asphyxial in origin, but doses of azide insufficient to give rise to a convulsive response might, after a variable period of stimulation and irregularity of breathing, bring about a depression and gradual failure of respiration; this was often complicated by terminal asphyxial convulsions.

Other actions

Sodium azide dilated the perfused bronchus of guinea-pigs and stimulated both isolated and intact intestine of rabbit, guinea-pig, and cat. In anaesthetized animals this stimulation of the gut, accompanied by flushing with arterial blood, was obvious: records of the movements of an intestinal loop and of the pressure within it revealed a transient relaxation of the muscle preceding the increase in activity. The isolated uterus of rat, rabbit, and guinea-pig was not affected by azide, but that of the cat was stimulated. In the pregnant cat under ether, however, this effect was negligible with amounts of azide which caused a prolonged fall in blood pressure: the urinary bladder contracted vigorously and expelled urine. There was no difference in the diuretic response of groups of rats injected with small doses of azide after receiving water by mouth and control animals not given azide, but if the dose of azide was increased the rats showed evidence of collapse and urine production ceased. Frequency of micturition as seen in intact animals given azide is not due to diuresis but to the action on the urinary bladder, probably a direct one. Azide is water soluble and was absorbed from all routes of administration. It did not discolour the blood with methaemoglobin as did nitrite: like nitrite it was not excreted unchanged in the urine, which failed to give the brown colour reaction with ferric chloride obtained *in vitro* with dilute sodium azide before injection or feeding.

Discussion

The actions of sodium azide on the cardiovascular system of mammals are similar to those of sodium nitrite. The effect of inhaled amyl nitrite on the systemic blood pressure as described by Bradford and Dean (1894) is similar to that of hydrazoic acid gas as described by Smith and Wolf (1904) and elaborated by Graham, Robertson, and Rogan (1948). That the tachycardia caused by nitrite is a reflex one following upon the fall in blood pressure was shown by Dossin (1911), whose findings with the action of nitrite on the isolated perfused heart are paralleled by those described above for azide. Loeb (1904) showed that nitrite is a coronary dilator, and Boyer, Wégria, and Green (1939) and Essex, Wégria, Herrick, and Mann (1940) confirmed this finding by modern techniques. The same action has been demonstrated with azide. Both nitrite and azide stimulate the respiration, but Heymans, Bouckaert, and Dautrebande (1931) attribute to the carotid sinus a larger part in the reflex stimulation of respiratory and cardiovascular mechanisms by nitrite than the present work indicates is played by the carotid sinus in the action of azide. Leech (1893) and Smith and Wolf (1904) have shown respectively that nitrite and azide inhibit the contractile power of isolated frog gastrocnemius muscle. According to Beams and Barlow (1932) nitrite causes a contraction and then a relaxation of isolated strips of rabbit gut in Locke's solution: azide causes a contraction *in vitro* and a relaxation followed by a contraction *in vivo*. Both substances dilate the perfused bronchi. Both are modified by metabolic processes in the body: their fate is as yet unknown.

The main differences lie in the greater direct effect of azide on the central nervous system, especially on the respiratory centre, the more powerful action of azide on the peripheral vascular bed, the absence of formation of methaemoglobin in the blood stream of animals receiving azide intravenously, and the proven effect of azide on various enzymatic processes in the living cell.

In human beings it has been shown that hydrazoic acid fumes lower systolic and diastolic blood pressure to a profound extent for some three to six hours with the production of a mild degree of headache.

The mechanism of action of azide is complex. Peripherally it relaxes the smooth muscle of blood vessels and bronchi while increasing the force of the cardiac contraction. This results in a greatly increased coronary flow accompanying a fall in systemic blood pressure. Centrally it stimulates the medulla, which results in a stimulation of respiration, an initial vagal inhibition of the heart, and a subsequent sympathetic stimulation of the heart. Azide also has a direct stimulant action on cardiac muscle which may play a part in this phenomenon. The resultant effect of these opposing forces is determined by the species and state of the animal, especially the initial blood pressure, the sensitivity of its nervous system and reflex mechanisms, the anaesthetic administered, and the dose and rate of administration of the azide. The larger doses of azide (mg. rather than μg. /kg.) powerfully affect the sympathetico-adrenal mechanism and cause a prolonged rise in systemic blood pressure and a great increase in cardiac force, probably brought about by a release of adrenaline from the suprarenal glands. The variable effects on the volume of the spleen and the leg are the result of these conflicting mechanisms. The activity of gut and bladder is increased, that of the uterus is little altered. Stimulation of the central nervous system, which may be severe

enough to cause a characteristic convulsive seizure, is in other cases followed by depression leading to asphyxia from respiratory failure.

Summary

Sodium azide, a neutral stable salt of hydrazoic acid, is a potent hypotensive agent which dilates peripheral blood vessels by direct action. It stimulates cardiac muscle and dilates the coronary vessels directly.

It affects the rate and force of the mammalian heart *in vivo* by stimulating the vagal and sympathetic cardiovascular mechanisms: such effects are produced centrally rather than by carotid sinus reflexes.

It stimulates respiration and in large doses produces generalized convulsions followed by respiratory depression.

It increases gut and bladder contractions but hardly affects the uterus.

The general action is similar to that of sodium nitrite, but azide is more powerful.

The LD50 i.p. in white mice was 28–34 mg./kg. when that of sodium nitrite was 168 mg./kg.

It does not produce methaemoglobin *in vivo* and is excreted as a metabolite which no longer gives a brown colour reaction when ferric chloride is added to the urine.

During the performance of this work the author held an I.C.I. Fellowship in Pharmacology. Expenses were defrayed by a grant from the Medical Research Council.

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Brit. J. Pharmacol. (1949), **4**, 7.

A KYMOGRAPHIC STUDY OF THE ACTION OF DRUGS ON THE LIVER FLUKE (*FASCIOLA HEPATICA*)

BY

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From the Department of Pharmacology, University of Birmingham

(Received July 24, 1948)

The difficulty of culturing parasitic worms *in vitro* remains the major limiting factor in the development of methods for studying anthelmintic* activity directly on the parasite. This is because of the difficulty of maintaining *in vitro* a suitable environment for the different parasites. This has been achieved in a partial sense by Chu (1940) for *Chlonorchis sinensis* and *Schistosoma japonicum*, by Lamson and Brown (1936) for *Ascaris*, and by Smyth (1948) for plerocercoid larvae of *Schistocephalus solidus* and *Ligula intestinalis*, but for many other parasites methods for obtaining the same conditions do not yet exist. In their search for material which might be likely to provide alternative means, the early workers neglected the phylogenetic relationships of the different parasitic worms. Two main groups of helminth parasites exist: the nemathelminthes and the platyhelminthes.

Owing to the work of Robello and Rico (1926) and Baldwin (1943), a method exists for the *in vitro* study of anthelmintic activity on nematodes. This method, moreover, has enabled a start to be made on the mode of action of drugs on nematodes. Von Schroeder (1885) and Trendelenberg (1916) both reported that santonin, which has a high therapeutic reputation as an anthelmintic against nematodes, appears to have no effect on the activity of the whole worm, when it is tested by observing the activity of whole ascaris *in vitro*. Baldwin, however, has suggested it may bring about incoordination of movement by virtue of a simultaneous stimulation of the body musculature (intermediate preparation) and depression of central nervous control (anterior preparation). Many *a priori* reasons exist for considering the effect of substances on the movement of the parasite to be a primary mode of action. Thus we have chosen to study the effect of drugs on the liver fluke as a representative platyhelminth worm by a kymographic technique of short duration. The present paper is, therefore, concerned with a method for studying the mode of action of drugs on trematodes, and the value of the liver fluke (*Fasciola hepatica*), as a preparation for screening substances for possible anthelmintic activity against the parasitic members of both phyla, is assessed.

Collection of parasites

The flukes were obtained from the bile ducts of bovine livers and were dissected out within half an hour of the death of the host before the liver cooled to room temperature. They were washed and subsequently placed in boiling-tubes containing Ringer's solution buffered (to *p*H 8.5) at 37° C., not more than two flukes to each tube. This was found necessary in order to prevent the flukes from attacking each other, which they did when large numbers were enclosed together. Preparations were made from these flukes within six hours of collection. Satisfactory flukes always showed rippling movements of the whole body, and movement by means of the suckers, for at least 24 hours.

Methods of recording movement

Kymograph tracings were made of the movements of a fluke suspended in Ringer's solution at 37° C., the fluke being under slight tension. The attachment for the recording arm and fixed point were by means of platinum hooks passed through the body wall posterior to the ventral sucker and close to the

* The term anthelmintic is used by us to indicate that the drug has been found effective for the cure of infestations of helminth parasites either in veterinary or clinical practice.

posterior end of the body. It was found essential to keep the fluke below the surface of the Ringer's solution while these attachments were being made in the shortest possible time. The fluke was then allowed 10 minutes to recover before recording was started, after which normal movements were recorded for 15 minutes.

Method of testing drugs

Drugs soluble in water were dissolved in Ringer's solution ; those which were precipitated in this medium were separately dissolved in water to which the saline constituents were then added as this delayed precipitation for long enough to make the test (santonin, kamala, umbelliferone, and coumarine). Insoluble drugs were emulsified by the method of Baldwin. The substances were tested initially at a high concentration (1 : 1,000) and, if active, at lower concentrations suggested by the response obtained. Finally the minimal effective concentration, as judged in most instances by the disappearance of activity within 45 min. after the addition of the drug, was obtained with at least four flukes. The absence of effect at 1 : 1,000 on at least two preparations was taken to indicate that the drug was without activity.

TABLE I

STIMULANTS

Drug	Nature of preparation	Effective concentration
Carbon tetrachloride	Solution	1 : 2,000–1 : 4,000*
Tetrachlorethylene	Emulsion	1 : 2,000–1 : 5,000*
Hexachlorethane ..	,,	1 : 20,000–1 : 5,000*
β-Naphthol ..	Solution	1 : 10,000–1 : 5,000*
p-Cymene ..	Emulsion	1 : 4,000*
Strychnine HCl ..	Solution	1 : 10,000
Coumarine ..	,,	1 : 2,000
Umbelliferone ..	,,	1 : 2,000
β-Phenylethylamine HCl	,,	1 : 5,000
Ephedrine HCl ..	,,	1 : 2,000
Tyramine HCl ..	,,	1 : 1,000
Amphetamine sulphate	,,	1 : 20,000–1 : 10,000
l-Amphetamine sulphate	,,	1 : 10,000
d-Amphetamine sulphate	,,	1 : 80,000

* Indicates that the drug possesses other types of action at higher concentrations or after acting for longer periods of time.

Results

Rhythmical activity was found in a high proportion of worms taken from bovine livers, but in general worms from sheep's livers could not be relied upon to give good rhythmical activity. Rhythmical activity, which was occasionally interrupted by short quiescent periods, showed a marked variation in amplitude from worm to worm, but, after the establishment of the rhythm in any one preparation, the amplitude and the tone remained approximately constant for at least 2 hours (Fig. 1).

Three types of drug activity have been found: stimulant, paralysant, and lethal.

Stimulant drugs

On addition of the substances with stimulant properties listed in Table I, some interference with the activity of the preparation occurred, and subsequently more rapid rhythmical movements were resumed with or without a change in amplitude and tone. There was also a marked difference in the duration of these effects. The two lactones, coumarine and umbelliferone, produced rhythmical movements of large amplitude and low frequency at low concentrations. These movements, however, gradually diminished within a period of half an hour, and were replaced by sustained contraction when high concentrations were tested. The stimulation produced by strychnine hydrochloride was characterized by increase in the frequency, reduction in the amplitude, and increase in the tone. The four amines all produced an increase in the amplitude and frequency after the initial contraction had subsided and produced some increase in the tone.

The chlorinated hydrocarbons with known anthelmintic potency and therapeutic value against

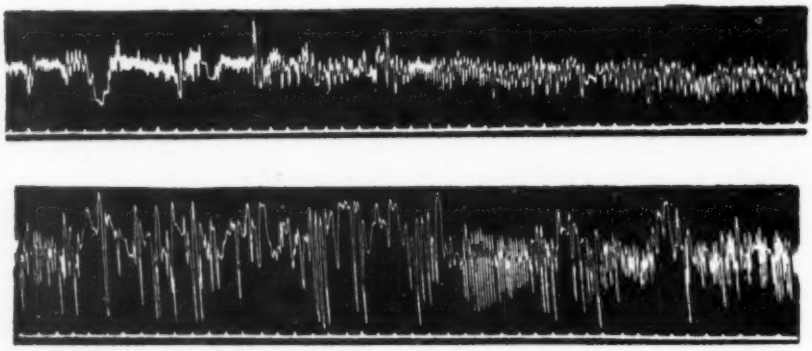
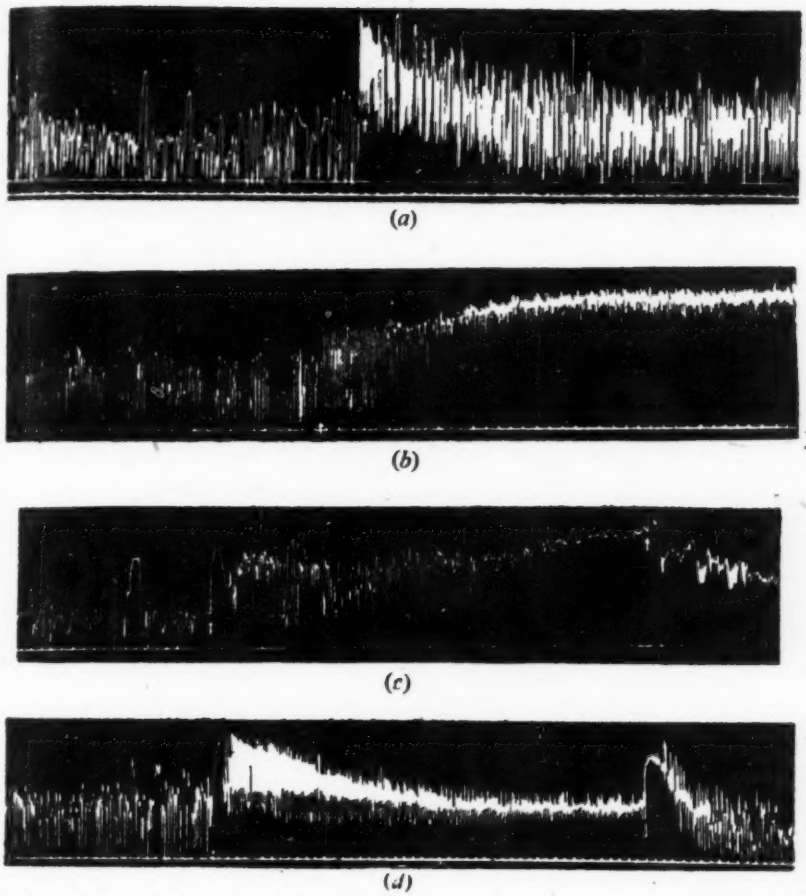


FIG. 1.—Two representative records of normal movement. (In all tracings upward stroke represents contraction. Time markings in minutes.)



(*a*)

(*b*)

(*c*)

(*d*)

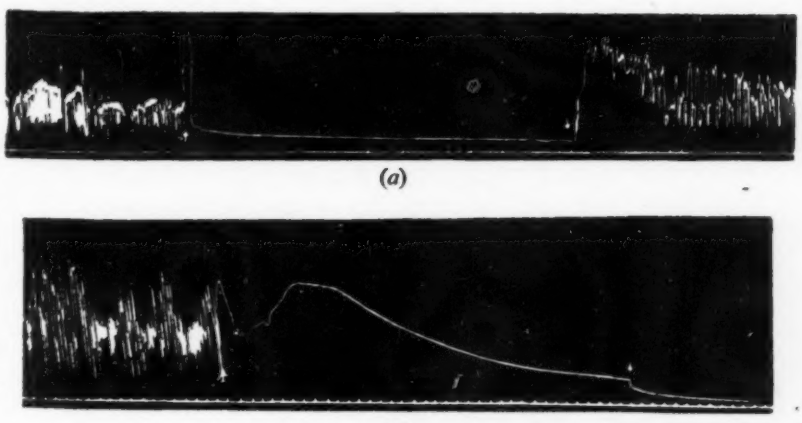
FIG. 2.—Stimulant drugs: Action on normal movement of: (*a*) amphetamine (1: 1,000); (*b*) tetrachlorethylene (1:5,000); (*c*) hexachlorethane (1: 20,000) followed by amphetamine; (*d*) coumarine (1: 1,000) followed by amphetamine.

infestations of the liver fluke all possess stimulant properties at low concentrations. This property was exhibited to a more marked degree by hexachlorethane (Fig. 2).

From the records it will be seen that the amines have a pronounced potentiating action on the normal rhythm, increasing both the amplitude and the frequency with only slight effect on the tone of the muscle. Moreover, amphetamine was the most powerful of these potentiating amines.

Paralysant drugs

Early on in the investigation it appeared likely that amphetamine might be used to bring back



(*a*)

(*b*)

FIG. 3.—(*a*) Paralysant action of pelletierine tannate (1 : 1,000) followed by response to amphetamine. (*b*) Lethal action of hexylresorcinol (1 : 5,000) followed by absence of response to amphetamine.

TABLE II

DRUGS WHICH PARALYSE THE PREPARATION BUT RHYTHMICAL CONTRACTION IS RESTORED BY AMPHETAMINE 1 : 5,000

Drug	Nature of preparation	Effective concentration
Nicotine	Solution	1 : 200,000
Carbaminoylcholine chloride (Doryl) ..	,,	1 : 20,000
Sodium amytal ..	,,	1 : 5,000
Arecoline HBr ..	,,	1 : 10,000,000
Oil of chenopodium	Emulsion	1 : 20,000–10,000*
Pelletierine tannate	Solution	1 : 2,000
Phenylurethane ..	Emulsion	1 : 2,000*
Santonin	,,	1 : 1,000
Kamala	Filtered saturated solution	*

* Indicates that the drug possesses other types of action at higher concentrations or after acting for longer periods of time.

TABLE III

DRUGS WHICH PARALYSE THE PREPARATION AND RENDER IT INSENSITIVE TO THE ACTION OF AMPHETAMINE 1 : 5,000

Drug	Nature of preparation	Effective concentration
Oil of chenopodium	Emulsion	1 : 5,000
p-Cymene ..	,,	1 : 1,000
Thymol	Solution	1 : 10,000
Hexylresorcinol ..	,,	1 : 10,000–1 : 5,000
β-Naphthol ..	,,	1 : 4,000–1 : 2,000
Carbon tetrachloride	Emulsion	1 : 1,000
Tetrachlorethylene	,,	1 : 1,000
Hexachlorethane	,,	1 : 1,000 (see text)
Ext. filix mas. ..	,,	1 : 5,000
Gentian violet ..	Solution	1 : 5,000
Phenylurethane ..	,,	1 : 1,000
Chlorbutol ..	,,	1 : 1,000
Kamala	Sat. solution	

normal rhythmical activity to preparations when the movement had been reversibly altered by a drug ; this might occur after inactivation by paralysant drugs or when the type of movement was markedly altered by other stimulant drugs. No such action would be expected after an effect which was to any extent irreversible, or after lethal drugs. It was therefore decided to add amphetamine sulphate in a concentration of 1 : 5,000 to the bath in the absence of the test substance, whenever the activity of the preparation had been obliterated or altered at the end of 45 min. This was done by replacing the solution of the test substance by Ringer's solution containing 1 : 5,000 amphetamine sulphate. By this means it was found possible to distinguish between drugs with paralysant action (listed in Table II, Fig. 3) and drugs with a lethal or probably lethal action (listed in Table III, Fig. 3). A large number of miscellaneous substances with no known anthelmintic action were tested and found to have no more than a transient action (Table IV, Fig. 4). The fluke musculature is relaxed to different degrees by parasympathomimetic drugs, except pilocarpine, and this relaxation is antagonized by amphetamine.

TABLE IV

DRUGS WHICH FAILED TO CAUSE COMPLETE PARALYSIS OF THE PREPARATION UP TO A CONCENTRATION OF 1 : 1,000 (MINIMUM 45 MIN.)

Phenothiazine	*d*-Tubocurarine chloride
Sodium tauroglycocholate	Yohimbine HCl
Ethyl alcohol (1%)	Atropine sulphate
Sulphanilamide	Hyoscine HBr
Sulphathiazole	Histamine acid phosphate
Penicillin (1,340 i.u./c.c.)	Cocaine HCl
Neosalvarsan	Quinine sulphate
Emetine HCl	Morphine HCl
Acetylcholine chloride	Caffeine
Acetyl-β-methylcholine chloride*	Guanidine HCl
Pilocarpine nitrate	Sodium bromide
Eserine sulphate*	Phenyl urea

* Transient depression

Lethal drugs

The chlorinated hydrocarbons which in low concentrations stimulate the fluke are lethal at concentrations ranging between 1 : 5,000 and 1 : 1,000. Hexachlorethane has exceptional properties which make it difficult to obtain known concentrations of it above 1 : 5,000 ; it is insoluble in water and sublimes below the boiling point of water. Thus, although sufficient substance was added to make an emulsion at 1 : 1,000 with alcohol some of the substance was precipitated. The emulsion made in this way, however, obliterated all movement in 90 min., after which amphetamine was without effect. The 1 : 5,000 solution

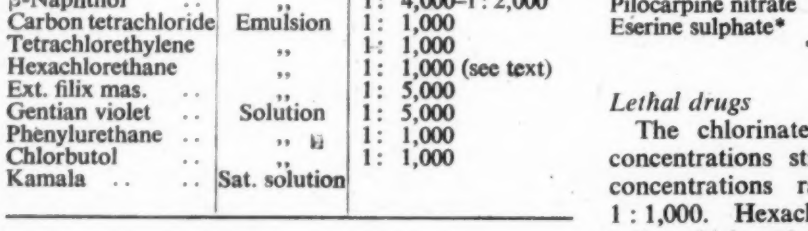


FIG. 4.—Absence of effect of morphine sulphate (1 : 1,000).

almost completely eliminated the activity, and correspondingly its action was only slightly affected by amphetamine. It would therefore appear to be a lethal drug in low concentrations, possibly with greater effect than the other chlorinated hydrocarbons. Ext. filix mas. is lethal at the same concentration in a shorter time. This is also true of gentian violet.

Ineffective substances

Phenothiazine was the only inactive anthelmintic with the exception of drugs active on blood flukes.

Discussion

The value of any particular approach to the study of anthelmintics can best be understood when it is realized that the host-parasite relationship is a special instance of the organism-environment relationship which forms the background to all biological studies. Veterinary practice possesses a variety of methods for combating parasitic infestations which act in one of four ways on the biological system comprising the host-parasite relationship. The first is by alterations in the relationship of the host to its environment; the second is by effecting changes in the internal environment of the host; the third is by affecting the relationship of the parasite to the internal environment of the host; and the fourth is by altering the internal environment of the parasite itself. The pharmacology of anthelmintics should be concerned with all four divisions, but in practice the first division is usually considered a separate field of study in the domain of ecology. Within the three remaining fields which are concerned with the administration of substances to infested animals or man the study of anthelmintics has been approached either as a chemotherapeutic problem or as if it were a pharmacological study on isolated organ systems of vertebrates. In fact both aspects must be integrated to provide a satisfactory approach to the problem. Chemotherapeutic tests are primarily effective for the detection of substances of therapeutic value, because micro-organisms are primarily dependent on a continuous process of reproduction for their distribution and for the location of the reproductive phase of their life cycle. Substances, therefore, which interfere with metabolic processes by whatever means are effective in reducing the distribution and influencing the location of micro-organisms. In parasitic worms, however, the distribution and the location of the adult reproductive worm are differentiated functions and depend on the separate physiological processes of reproduction and movement. Distribution is largely dependent on reproduction though movement plays some part in it, but the location of the adult is almost wholly dependent on movement which appears to be influenced by environmental factors. Elimination of the parasite, therefore, is dependent upon the drug affecting either the mechanism by which location is achieved and maintained or by suppressing reproduction. It is at present impossible to study the effect of drugs on reproduction *in vitro* because of difficulties of culturing them, but certain pharmacological techniques have been applied to the study of movement in parasitic worms, as in the present paper.

We require to know the effect of any drug on the components of the fluke's movement and on the co-ordination of these in its behaviour. Provided that the same effects are produced *in vivo* as *in vitro* we can then hope to get some idea of the possible modes of action of anthelmintics which affect movement. At present, we have only taken a step in this direction. The kymographic technique, which we have used, has enabled us to distinguish three different types of action affecting movement from which a classification of drugs can be made.

Some drugs are stimulants as defined earlier; these are the amines, the lactones, and strychnine. Other drugs are stimulants at low and lethal at higher concentrations—namely, the halogenated hydrocarbons, *p*-cymene and β-naphthol. This combined action may well be the reason why the halogenated hydrocarbons are effective on the liver fluke itself *in vivo*, whereas gentian violet, which *in vitro* is more lethal but has no stimulant action, is ineffective.*

Tetrachlorethylene has been shown by Rogers (1944) to have a stimulant effect on *Nippostrongylus muris*; this appears to be an exaggeration of normal movement since it caused the parasite to leave the mucous lining of the intestine. Halogenated hydrocarbons, therefore, as a group are likely to be stimulant to both phyla. Baldwin's (1943) technique using segments of *Ascaris* would appear to be inadequate, therefore, to reveal all types of stimulant action probably because his preparations are only part of the whole animal.

Finally there are drugs with paralysant action at low concentrations which become lethal as the concentration is raised—e.g., phenylurethane and oil of chenopodium.

Because major systematic differences such as those between phyla are most likely to be associated

*Private information from Dr. E. L. Taylor.

with major biochemical differences it is to be expected that comparison between members of the two differential phyla will reveal the presence or absence of selective* action within the helminths when tested *in vitro*. It is therefore worth while comparing our results with those of Baldwin.

As might be expected, the liver fluke is killed by all the protoplasmic poisons which affect *Ascaris*. Moreover, such comparison demonstrates that the liver fluke is affected by lower concentrations of the drugs which are lethal to both preparations. The liver fluke is also affected by some drugs which are not effective on *Ascaris*—i.e., the fluke preparation is sensitive to a wider range of known anthelmintic drugs than the *Ascaris* preparation. It should here be noted that Baldwin's preparation is insensitive to the action of gentian violet, which is active on threadworms *in vivo*. This may be due to an *in vitro* selective action of gentian violet on threadworms, but it might equally well be due to the fact that Baldwin's preparation does not allow direct access of the substance to the gut of the *Ascaris* preparation. The evidence available suggests that the cuticle is a more effective, and probably also a more selective, barrier to the penetration of drugs in *Ascaris* than in the liver fluke. Comparative tests on flukes with intact cuticles, and cuticles pierced at the point of attachment, reveal that the intact fluke is only slightly more resistant to all the drugs which we have tested. On the other hand some drugs are without effect on *Ascaris* because of fundamental differences in the neuromuscular mechanism. An explanation of the different responses to pelletierine and arecoline should be sought in one or both of these mechanisms. By contrast, those drugs which are effective for the elimination of cestodes from the intestine *in vivo* are also active on the liver fluke *in vitro*; a result which is consistent with the systematic relationships of the two parasites. The paralysant action of arecoline, which relaxes the muscle, parallels the results obtained by Betham (1946) on segments of *Taenia*.

The kymographic test of Betham does not allow any strict comparison to be made between the action of drugs on trematodes and cestodes, owing to the restricted use to which it was put. The only drug used as an anthelmintic with selective action directly on nematodes is santonin. Pelletierine and ext. filix mas., because they are active only on the fluke *in vitro* but not on *Ascaris* and because they are used exclusively against cestodes in the intestine, are likely to prove selective for platyhelminthes. Gentian violet, on the other hand, although it does not affect Baldwin's preparation, does eliminate nematodes (threadworms) from the intestine and is therefore almost certainly active on both phyla. In this connexion it should be noted that, of the two lactones which are stimulant to the fluke, coumarine paralyses *Ascaris* (posterior segment) and umbelliferone is without effect. This is also true of strychnine. The mode of action of phenothiazine and drugs active on blood flukes is still obscure.

Stephenson (1947) measured the effect of carbon tetrachloride, tetrachlorethylene, and gentian violet on the survival time of the liver fluke *in vitro* and was unable to detect any action of the chlorinated hydrocarbons, but obtained some effect with gentian violet, thus demonstrating that his method was less sensitive than the one under discussion. We attribute this to the difference in viability of the preparation in the two types of test. In an acute test, using as a measure of activity the effect on movement, the viability of the preparation is assured. This is not so in a test based on survival time, as the viability of the preparation falls off rapidly towards the end of the experimental period when the preparation is required to be most sensitive to differences in activity of the drugs under test. Moreover, the method led to paradoxical results in the hands of Chu (1940), who showed that *Chlonorchis sinensis* survived longer in higher concentrations of certain drugs (e.g., methylene green and trypan blue) than at lower concentrations; that in effect there was a reverse relationship between dose and effect which was attributed to a reduction in oxidation rate preventing the parasite *in vitro* from poisoning itself with its own excreta.

The comparisons we have made suggest that the majority of drugs used as anthelmintics are protoplasmic poisons acting on both phyla and also on the host. The apparent selectivity in the anthelmintic activity of the protoplasmic poisons *in vivo* does not therefore reflect a selective action on different parasites but rather the importance of different biochemical and physiological conditions in the immediate neighbourhood of the parasite affecting the action of these poisons. This emphasizes the need for tests capable of distinguishing the selective action of drugs *in vitro*, if safer anthelmintics are to be found. This does not mean that we require to test drugs on isolated members of each species but that we require to assess the significance of systematic differences revealed by tests on representative members of each phyla.

* The word selective is used by us to indicate that drugs act exclusively on a few parasites. It is qualified by the adverbs *in vitro* and *in vivo*. (Specificity should be reserved for testing the action of drugs on different parts of an organism. In this way its use in this field is consistent with the rest of pharmacology.)

Summary

1. It is possible to obtain rhythmical kymographic records from fresh bovine flukes suspended in Ringer's solution at a *p*H range from 6.5–8.5. These movements are maintained for a period of at least 2 hours and frequently as long as 6 hours.

2. The effect of known anthelmintics and of a number of other drugs, particularly those affecting the neuromuscular mechanism of vertebrates, have been tested on this preparation by allowing them to act for a maximum period of 45 min.

3. At the end of this period the addition of amphetamine restores rhythmical activity provided the drug has had only a paralysant action on the fluke. In this way it has been possible to distinguish between stimulant, paralysant, and lethal drugs.

4. It is suggested that the possible reason why the chlorinated hydrocarbons are so effective as anthelmintics against *Fasciola hepatica* is that they combine the stimulant action at low with the lethal at higher concentrations. A number of other anthelmintics have been shown to have other types of combined action.

5. Comparison with the similar test on *Ascaris* segments (Baldwin, 1943) reveals that the liver fluke as a representative of the platyhelminthes is sensitive to all the drugs which affect *Ascaris* and in addition to umbelliferone, pelletierine, extract filix mas., and gentian violet. The significance of this comparison is discussed.

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SOME PHARMACOLOGICAL ACTIONS OF PALUDRINE

BY

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Since the discovery of paludrine by Curd, Davey, and Rose (1945), few workers have studied its general pharmacological properties, though its low toxicity in comparison with many antimalarials is well known. Spinks, Tottey, and Maegraith (1946) found that the absorption, distribution, and excretion of paludrine in the rat resembled that of mepacrine. Spinks (1947) investigated the fate of paludrine given to rats and mice by mouth and found that it was rapidly absorbed from the stomach; a low recovery from the faeces and urine suggested that it was broken down in the body. Butler, Davey, and Spinks (1947) showed that the acute toxicity of paludrine varied both with the species and the method of administration. The intravenous or intraperitoneal injection of paludrine into rats and mice was followed by delayed deaths, the animals dying up to 24 hours after the injection; this was the more surprising in view of the fact that a closely related substance, 4430, did not exhibit this property. At first it was thought that these delayed deaths were caused by unusually prolonged retention of paludrine in the blood, but this was later disproved by measuring blood concentrations. Chicks given paludrine intravenously died within 15 min. or not at all, and from this and other evidence it was concluded that the metabolism of paludrine in chicks (and probably man) was different from that in rats and mice.

Hughes and Schmidt (1947) and Hughes, Schmidt, and Smith (1947a, b) also studied the absorption, toxicity, and excretion of paludrine in various animals. In the dog, paludrine induced copious salivation, loss of appetite, extreme cachexia, and cardiac arrhythmia. Feeding tests were performed in which paludrine was mixed with the food: the dogs refused to eat, and died from starvation. Chen and Anderson (1947) investigated the toxicity and then described in more detail the effects of paludrine in the body. In the anaesthetized or pithed cat, paludrine caused a temporary fall of the blood pressure. The respiratory rate was increased, but the depth diminished. Isolated loops of rabbit intestine were relaxed by paludrine and the isolated uterus of the guinea-pig was stimulated. Contractions of isolated intestine of the guinea-pig, induced by histamine, were inhibited. Innes (1947) showed that intramuscular injections of paludrine lactate caused necrosis, haemorrhage, oedema, and inflammatory exudate, with local involvement of nerves and vessels; this was most intense about 12 days after the injection. It is now generally believed that some metabolic product of paludrine is responsible for its antimalarial activity (Hawking and Perry, 1948), but so far attempts to identify this product have failed.

Experimental Results

Toxicity.—The acute intravenous toxicity of paludrine in mice of weights between 18 g. and 25 g. was studied. The LD50 was found to be 22 mg./kg., which agrees well with the results of other workers. As Butler, Davey, and Spinks (1947) observed, the toxicity of paludrine was sometimes delayed, and the figure for the LD50 given above was calculated after observing the mice for 72 hours after the injection. A possible explanation of the delayed toxicity was that paludrine interfered with some esterase mechanism in the animal. Blaschko, Chou, and Wajda (1947) showed that paludrine had an affinity for pseudocholinesterase and for the benzoylcholinesterase of guinea-pig's liver.

It was thought that the simultaneous injection of prostigmine, a very strong inhibitor of cholinesterase, might significantly alter the toxicity of paludrine. The intravenous toxicity of prostigmine in mice was therefore determined, and from the dose/response curve two doses of prostigmine were chosen; one which killed no mice (90 μg./kg.) and one which killed about 30 per cent of the mice (180 μg./kg.). Each of these doses was then injected simultaneously with the LD50 dose of paludrine (22 mg./kg.). The results are summarized in Table I.

TABLE I

INTRAVENOUS TOXICITY OF PALUDRINE, PROSTIGMINE, AND MIXTURES OF THE TWO

Group	Dose of drug	No. of mice used	Instant deaths	Delayed deaths			Total deaths	% Mortality
				0–24 hr.	24–48 hr.	48–72 hr.		
A	Prostigmine 90 μg./kg. ..	15	0	0	0	0	0	0
B	Prostigmine 180 μg./kg. ..	30	8	0	0	0	8	26.6
C	Paludrine 22 mg./kg.	30	0	8	4	4	16	53.4
D	Paludrine 22 mg./kg. + Prostigmine 90 μg./kg.	30	8	5	5	3	21	70
E	Paludrine 22 mg./kg. + Prostigmine 180 μg./kg.	30	29	0	0	0	29	96.7

Table I shows that the injection of prostigmine simultaneously with paludrine increased the immediate toxic effect. The immediate deaths, taken as a percentage of the total deaths, were increased from 0 per cent to 38 per cent by the addition of a non-toxic dose of prostigmine (90 μg./kg.), and to 100 per cent by a dose of prostigmine (180 μg./kg.), which alone killed about 27 per cent of animals.

Effect of paludrine on the cardiovascular system

Action on blood pressure and vessels.—In the anaesthetized animal an intravenous injection of paludrine usually caused a transient fall in blood pressure. In the perfusion of the dog's hind leg with heparinized blood, paludrine was shown to have a vasodilator action ; 2 mg. injected into the perfusion cannula decreased the pressure and increased the outflow of blood. This vasodilatation was diminished by neoantergan. The same effect was observed on the blood pressure of a cat anaesthetized with chloralose. Both paludrine (4 mg.) and histamine (10 μg.) caused a fall in blood pressure: this fall was reduced by the injection of benadryl. Thus the antihistamine agents, neoantergan and benadryl, reduced the vasodilatation caused by paludrine.

Paludrine antagonized the action of adrenaline on both the dog's perfused hind leg and the cat's blood pressure. The latter effect is shown in Fig. 1. In both preparations the vasoconstriction produced by adrenaline was reduced by paludrine.

Action on heart muscle.—The refractory period of the auricle was lengthened by paludrine. The compound was compared with quinidine by Dawes's method on the electrically stimulated rabbit auricles (Dawes, 1946), and it was found to be approximately one-eighth as active as quinidine (Table II).

On the isolated perfused cat heart (Langendorff preparation), paludrine (10–500 μg.) inhibited the amplitude and rate of beat according to the dose.

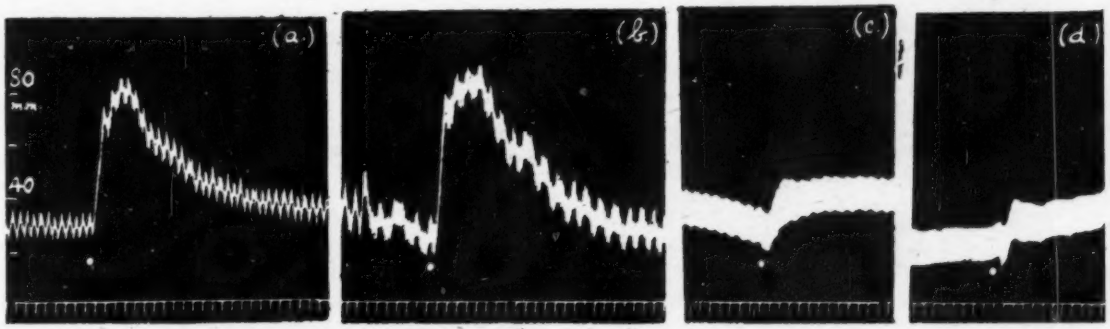

FIG. 1.—Blood pressure of chloralose cat from carotid artery. (*a*) Adrenaline (5 μg.); (*b*) adrenaline (5 μg.). Between (*b*) and (*c*) paludrine (80 mg.) was infused intravenously. (*c*) Adrenaline (5 μg.); (*d*) adrenaline (10 μg.). Time 10 sec.

TABLE II

PERCENTAGE LENGTHENING OF THE REFRACTORY PERIOD OF ISOLATED RABBIT AURICLES

The figures are averages of those from five experiments.

Paludrine		Quinidine	
Dose, mg.	% increase of refractory period	Dose, mg.	% increase of refractory period
1	4	0.25	9
2	9	0.5	16
4	14	1.0	23

The inhibition was accompanied by coronary dilatation.

Paludrine depressed both the contractility and the rate of beat of isolated rabbit auricles. At the same time the normal inhibitory action of acetylcholine was changed to a stimulation. If paludrine was allowed to act for a longer time the auricles stopped beating: acetylcholine would then restart the contractions. This action is described in more detail by Burn and Vane (1949). The isolated frog heart was also depressed by paludrine.

Action on striated muscle

On the isolated rectus abdominis of the frog paludrine had three distinct actions. When contractions of the muscle were obtained with acetylcholine, paludrine in very low concentration (5.0×10^{-7}) was found to augment these contractions. In concentrations higher than 10^{-6}, paludrine inhibited the action of acetylcholine. Finally, in concentrations of over 10^{-4}, paludrine alone caused the muscle to contract; the response to the same concentration of paludrine increased with

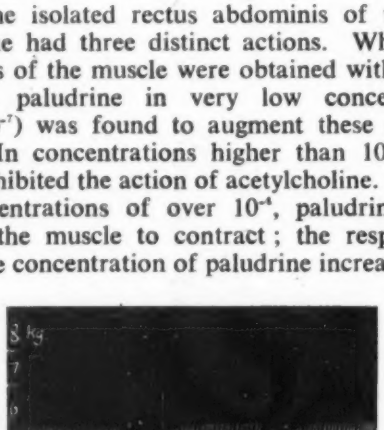

FIG. 2.—Cat sciatic-gastrocnemius preparation. Contractions of muscle evoked by maximal stimulation of the sciatic nerve by square waves at 24 shocks per min. Effect of two doses of paludrine injected into the artery.

successive applications. Thus, paludrine had a biphasic action on contractions evoked by acetylcholine; in small concentrations it augmented the contractions; in higher concentrations it depressed them.

Cat sciatic-gastrocnemius preparation. — The muscle twitch evoked by single maximal nerve volleys was depressed by paludrine (2–20 mg.) injected into the arterial blood stream (Fig. 2). This curariform action was also observed on the isolated phrenic nerve-diaphragm preparation of the rat.

Action on smooth muscle

Intestinal movements (in situ).—The intestinal movements of a cat, anaesthetized with chloralose, were recorded by a balloon tied into the duodenum. Alterations in volume of the balloon were recorded by a water manometer and piston recorder. A typical record is shown in Fig. 3; the upper tracing is the record of the intestinal movements; the lower one that of the blood pressure. The natural movements of the duodenum in this experiment were quite vigorous (*a*). An intravenous infusion of paludrine (1.3 mg./min.) lowered the tone of the muscle and abolished the natural movements. The paludrine infusion was stopped and the natural movements of the gut slowly returned; (*f*) shows the effect of a single injection of paludrine; the intestinal movements were stopped almost immediately. This effect was confirmed in three similar experiments. The blood pressure record in this experiment showed an interesting effect; the heart was irregular and often dropped beats (see *a* and *f*). The slow infusion of paludrine eliminated most of these, and after the single injection of paludrine all the irregularities disappeared.

Intestinal movements after vagal stimulation.—In some cats prepared for the above experiment the natural movements of the gut were small. In these animals the right vagus nerve was exposed in the neck, cut, and the peripheral end stimulated by an induction coil for 30 sec.; this produced a burst of motility in the duodenum. An intravenous infusion of paludrine decreased the response of the intestine to vagal stimulation; the activity recovered when the infusion was stopped.

Isolated intestine.—The contractions of isolated guinea-pig ileum elicited by acetylcholine were inhibited by paludrine, and so were the contractions elicited by histamine. The inhibitions of the contractions were of the same order in each case. Paludrine, in a concentration of 2×10^{-6}, decreased the natural tone of isolated rabbit duodenum; at

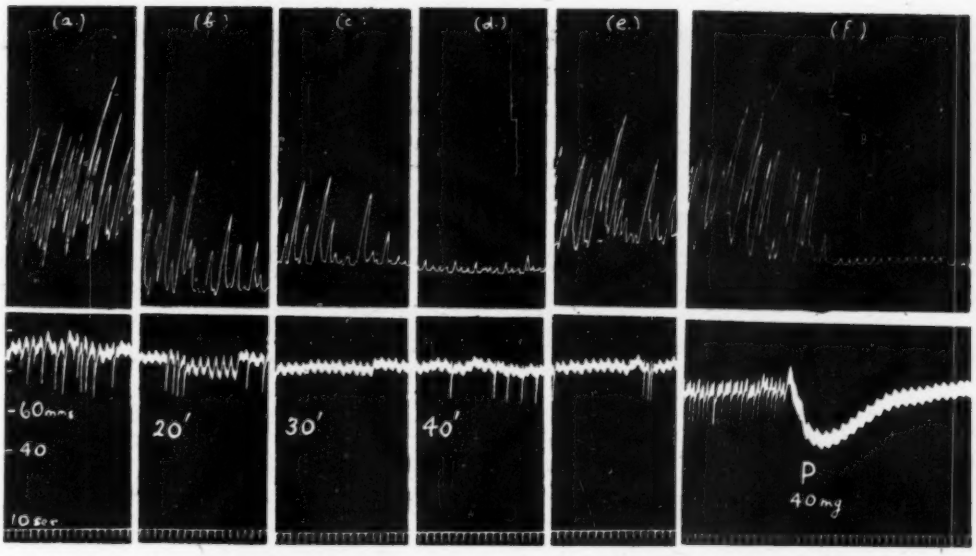

FIG. 3.—Chloralose cat. Top: intestinal movements, recorded by a balloon in the duodenum. Bottom: blood pressure from carotid artery. (*a*) Natural movements; infusion of paludrine started at 1.3 mg./min.; (*b*) 20 min. after start of infusion; (*c*) 30 min.; (*d*) 40 min. Infusion stopped (52 mg. infused). Activity began to return; (*e*) 10 min. later. Eventually normal activity returned, as shown in (*f*) (1 hour later). A single injection of paludrine (40 mg.) again stopped the movements. Note effect on blood pressure and dropped beats. Time 10 sec.

the same time it augmented the response of the muscle to adrenaline.

Splenic volume.—The volume of the spleen of the cat was recorded by means of a plethysmograph attached to a piston recorder. Paludrine injected intravenously had little effect on the blood pressure, but dilated the spleen. This was confirmed in six other experiments.

Action on bronchioles.—The bronchial tone was recorded by the method described by Konzett and Roessler (1940), and Emmelin, Kahlson, and Wicksell (1941), using the recorder described by Halpern (1942).

The guinea-pig was anaesthetized with urethane, and cannulae were inserted into the left jugular vein and the trachea. The lungs were artificially respired by a pump; the excess air was measured by the apparatus referred to above, so that increased excursion of the recorder on the smoked paper indicated broncho-constriction.

Injections of paludrine (1–20 mg.) into the jugular vein had in themselves no effect, but greatly enhanced the broncho-constriction caused by histamine. Fig. 4 shows this effect. Histamine (2 μg.) produced regular constrictions of the bronchioles: the injection of paludrine (10 mg.) increased the histamine effect (*a*). Paludrine (20 mg.), whilst having no effect by itself, increased the histamine constriction so much that the guinea-pig died (*b*).

Respiration

Rabbits anaesthetized with urethane were used. Respiration was recorded by Gaddum's method (1941). Paludrine (8–20 mg.) reduced the respiration, but the depression did not last as long as the fall in blood pressure which accompanied it.

In further experiments a section of the right vagus nerve was exposed in the neck. The nerve was cut and the central stump was stimulated by an induction coil for 5 sec. once every minute. This produced a constant inhibition of the respiration as shown in Fig. 5*a*. Paludrine (8 mg.) injected intravenously caused a transitory depression of the respiration, after which the vagal effect was abolished. The stimulation once every minute was continued and the vagal depression gradually returned to its normal value in about 20 min. In another experiment in which paludrine (4 mg.) was injected, the effect reached its maximum at about 10 min. after the injection. In both these rabbits, as in four other experiments which gave similar results, the blood pressure remained constant, except for a small transient fall on injection of the paludrine.

Ganglionic transmission

The superior cervical ganglion of the cat was perfused with Ringer-Locke solution by the method of Kibjakow (1933). Stimulation of the

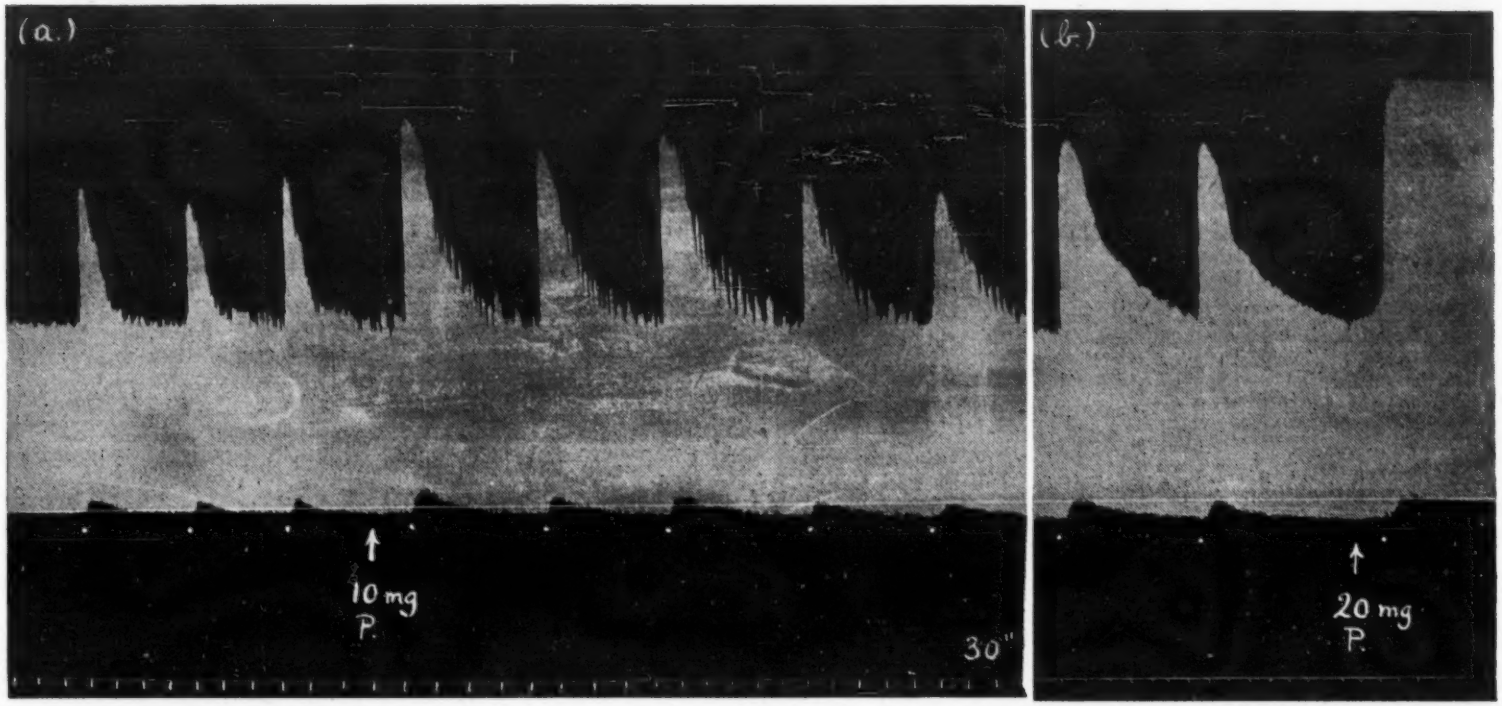

FIG. 4.—(*a*) Record of constrictions of guinea-pig bronchioles induced by histamine (2 μg.) intravenously injected at dot. At arrow paludrine (10 mg.) was injected. It had no action by itself, but potentiated the histamine response. (*b*) Constrictions caused by histamine (3 μg.). At arrow paludrine (20 mg.) was injected. This had no effect, but the next histamine injection killed the animal. Time 30 sec.

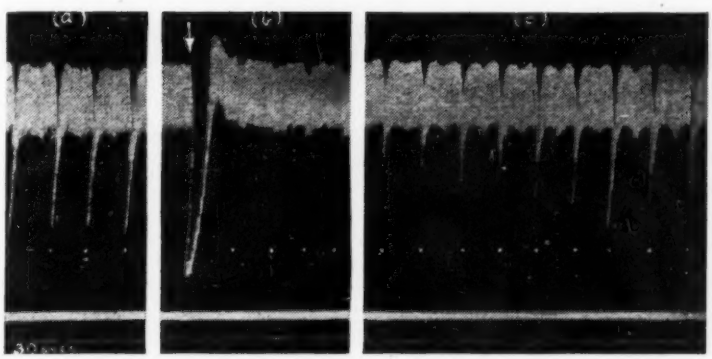

FIG. 5.—Respiration of rabbit recorded by Gaddum's method. (*a*) Central end of cut vagus nerve stimulated for 5 sec. once every minute. (*b*) Paludrine (8 mg.) injected at arrow. This caused transient depression of respiration. Effect of vagal stimulation abolished. (*c*) Effect of stimulation gradually returning, the first stimulation being 11 min. after injection of paludrine. Time 30 sec.

preganglionic fibres produced a contraction of the nictitating membrane: this was recorded on smoked paper by a lever with a frontal writing point. Injection of paludrine into the infusion cannula depressed the response of the nictitating membrane (Fig. 6); this appeared with doses of paludrine exceeding 200 μg.

Action on secretions

The action of paludrine on salivary secretion in cats was compared with that of atropine by the method of Bülbring and Dawes (1945). Although paludrine (4–16 mg.) gave a very transient inhibition, the inhibition was never as large as that produced by atropine (2 μg.).

The antidiuresis evoked by pituitary (posterior lobe) extract in rats was slightly prolonged by paludrine (10 mg./kg.) when injected with the pituitary extract; the method described by Burn (1931) was used.

Paludrine was found to inhibit the histamine-induced gastric secretion in cats, and also the gastric secretion evoked by a test meal in man. This effect is described in other papers (Burn and Vane, 1948; Vane, Walker, and Wynn Parry, 1948).

DISCUSSION

Late deaths after the intravenous injection of paludrine have been observed in rats and mice but not in chicks (Butler, Davey, and Spinks, 1947). It is interesting to note that late deaths also occurred after the oral administration of pamaquin, certuna, and mepacrine to fowls, and that the nervous symptoms which accompanied the delayed deaths prevented ingestion of food, the deaths sometimes being hastened by starvation (Köhlschütter, Zipf, and Triller, 1943). Paludrine given to rats, mice, dogs, and monkeys in sublethal doses also diminished the intake of food, as described by Hughes, Schmidt, and Smith (1947b). Thus, delayed deaths and loss of appetite seem to be common properties of the synthetic antimalarial drugs pamaquin, certuna, mepacrine, and paludrine.

Late deaths might also be due to interference with some enzyme system. Blaschko, Chou, and Wajda (1947) found that certain cholinesterases were inhibited by paludrine; the experimental results described in this paper show that when prostigmine was injected simultaneously with paludrine into mice the immediate deaths were increased and the delayed deaths decreased. This would be expected if the deaths were due to inhibition of the esterase by paludrine.

On the other hand, in most of its actions paludrine was found to antagonize the effect of vagal stimulation or of acetylcholine. The action of

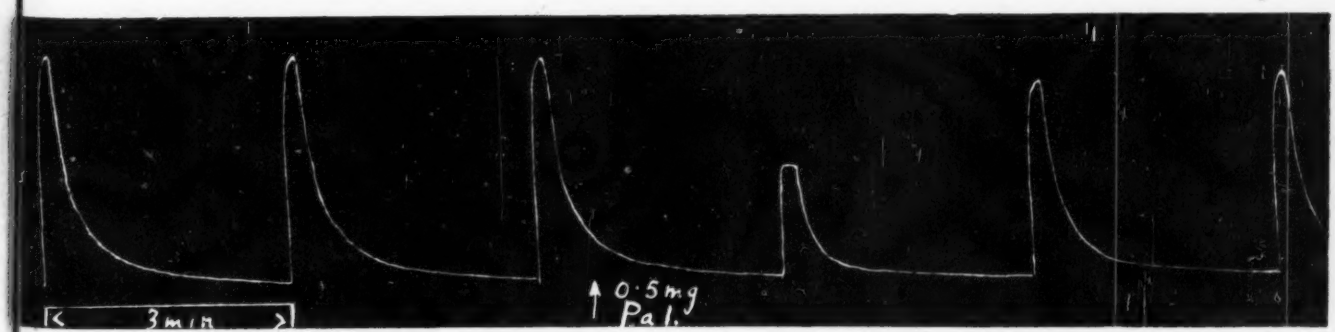

FIG. 6.—Perfusion of superior cervical ganglion. Stimulation of preganglionic fibres produced contractions of the nictitating membrane shown in tracing. Paludrine (0.5 mg.) injected into the perfusion fluid partially blocked the transmission.

acetylcholine was inhibited on the cat intestine *in situ*, on the isolated guinea-pig ileum, on the isolated rabbit auricles, and on the isolated frog rectus muscle. In smooth muscle the natural tonus was reduced. Quinine, quinidine, cinchonine, pamaquin, and mepacrine were found by Keogh and Shaw (1943, 1944) first to stimulate, and then relax, isolated rat's intestine. The same authors also showed that the depressor response of the cat's blood pressure to acetylcholine was reduced by quinine. Hiatt, Brown, Quinn, and Macduffie (1945) found that quinine, quinidine, and cinchonine all inhibited the action of the vagus on the heart.

Dawes (1946) discussed the relationship between the inhibitory action of drugs on the acetylcholine response and their effect in prolonging the refractory period of the heart. Paludrine has been found to lengthen the refractory period and to impair the contractility of isolated rabbit auricles. Here, too, the other antimalarial drugs have a similar effect. Quinine, mepacrine, and pamaquin decreased the heart rate and lengthened the conduction time as shown by the e.c.g. in man and in dogs (Molitor, 1941). The lengthening of the refractory period by quinidine, and to a lesser extent by quinine, is well known. Smith and Stoekle (1946) showed that mepacrine impaired the contractility of the heart.

A curariform action of paludrine has been demonstrated on the perfused superior cervical ganglion, on the cat sciatic-gastrocnemius preparation, and on the rat phrenic nerve-diaphragm preparation. Harvey (1939) described a curariform action of quinine on the neuromuscular junction

Paludrine in large doses has been shown to antagonize the vasoconstriction caused by adrenaline in the dog's perfused hind leg and on the cat's blood pressure. Keogh and Shaw (1943, 1944) found that quinine in large doses reversed the action of adrenaline on the cat's blood pressure.

Molitor (1941) showed that mepacrine and pamaquin produced vasodilatation, as does quinine. In this work it has been shown that paludrine also produces vasodilatation in the dog's perfused hind leg and in the cat. The dilatation was partially abolished by the antihistamine agents, neoantergan and benadryl. This, with the work of MacIntosh and Paton (1947), who found that certain biguanides and amidines released histamine from muscle, suggested that paludrine may also release histamine from the tissues. The relationship between paludrine and histamine is very puzzling: whereas paludrine inhibited gastric secretion evoked by histamine and reduced the response of isolated guinea-pig ileum to histamine, it potentiated the action of histamine on the guinea-pig lungs. On the other hand the antihistamine agents, benadryl and neoantergan, which abolish the effect of histamine in most tissues but potentiate the gastric secretion caused by histamine (Wood, 1948), reduced the action of paludrine on the systemic vessels.

Summary

Certain pharmacological actions of paludrine are described.

1. In most experiments, paludrine antagonized the action of acetylcholine or of vagal stimulation: contractions of the isolated frog rectus muscle and guinea-pig ileum were inhibited by paludrine and the normal action of acetylcholine on isolated rabbit auricles was abolished. The effects of vagal stimulation on the cat intestine and rabbit respiration were also reduced, as was the natural tonus of the intestine.

2. As with other drugs which antagonize acetylcholine, paludrine lengthened the refractory period of auricular tissue. It also had a curariform action on the cat sciatic-gastrocnemius, the rat phrenic nerve-diaphragm, and the perfused superior cervical ganglion preparations.

3. Paludrine reduced or abolished the vasoconstrictor action of adrenaline in the dog's perfused hind leg and on the cat blood pressure.

4. Paludrine caused vasodilatation of the perfused dog hind leg and in the cat. This dilatation was reduced by injection of antihistamine agents, which suggested that paludrine might release histamine from the tissues. If this is so, then the relationship between histamine and paludrine is difficult to understand, for, whereas paludrine potentiates the constrictor effect of histamine on the guinea-pig lungs, it inhibits histamine-induced gastric secretion in cats. It also inhibits contractions of isolated guinea-pig ileum evoked by histamine.

5. The delayed toxicity observed in mice after the intravenous injection of paludrine was changed by the simultaneous injection of prostigmine, which increased the proportion of immediate deaths.

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THE ACTION OF SYNTHETIC CURARIZING COMPOUNDS ON SKELETAL MUSCLE AND SYMPATHETIC GANGLIA BOTH NORMAL AND DENERVATED

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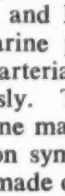
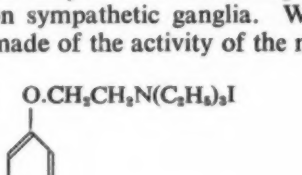
A recently synthesized substance F2559 with curarizing properties was shown by Bovet, Depierre, and de Lestrange (1947) to differ from *d*-tubocurarine in its effect on the blood pressure. An intravenous dose of tubocurarine chloride which caused muscular paralysis in the dog lasting several hours also caused a fall in general blood pressure, but a dose of the synthetic compound with the same curarizing action did not depress the blood pressure. The fall of blood pressure after an injection of tubocurarine may be caused in two ways. Firstly, it may be due to the release of histamine which was first observed by Anrep and his co-workers (Alam *et al.*, 1939). However, Grob, Lilienthal, and Harvey (1947) showed that in man tubocurarine produced vascular changes only after intra-arterial injection and not when given intravenously. The second mechanism by which tubocurarine may lower the blood pressure is by its action on sympathetic ganglia. When a comparison was made of the activity of the mono-, di-, and tri-quaternary ammonium salts shown below, of which the substance mentioned above is a member, it was found that the compound with the strongest action on the blood pressure had the weakest curarizing action on skeletal muscle, whereas the compound with the strongest action on the muscle had the weakest action on the blood pressure. Moreover, Depierre (1947) showed that the ratio of doses of these compounds for blocking transmission in the superior cervical ganglion was similar to that for lowering blood pressure. It appeared interesting to make a more detailed investigation of the action of these three compounds on the normal and denervated sympathetic ganglion and to compare it with their action on normal and denervated skeletal muscle.

F2512 

β-diethylaminoethoxybenzene ethiodide

F2557 

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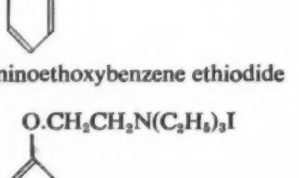
F2559 

1 : 2 : 3-tri-(β-diethylaminoethoxy)benzene triethiodide

Experimental Results

(1) *Effect on the response of the nictitating membrane to preganglionic sympathetic nerve stimulation in the perfused superior cervical ganglion*

Method.—Cats under chloralose were used. The ganglion was perfused with Locke's solution according to Kibjakow's (1933) method, modified by Feldberg and Vartiainen (1934). The preganglionic nerve was stimulated maximally at a rate of 16 per sec. for 15 sec. every 4 min. The compounds were injected into the arterial cannula one minute before each stimulation.

Results.—Depierre's finding was confirmed that F2559 was very weak in depressing synaptic transmission, the lowest dose having some effect being 1 mg. in one experiment whereas in another as much as 10 mg. was required. On the other hand, F2512 was found to be nearly as strong as *d*-tubocurarine chloride, acting in doses from 40 to 200 μg. Doses of F2557 causing synaptic depression were intermediate between those of the two other compounds. In six perfusions the average doses depressing synaptic transmission were 5 mg.

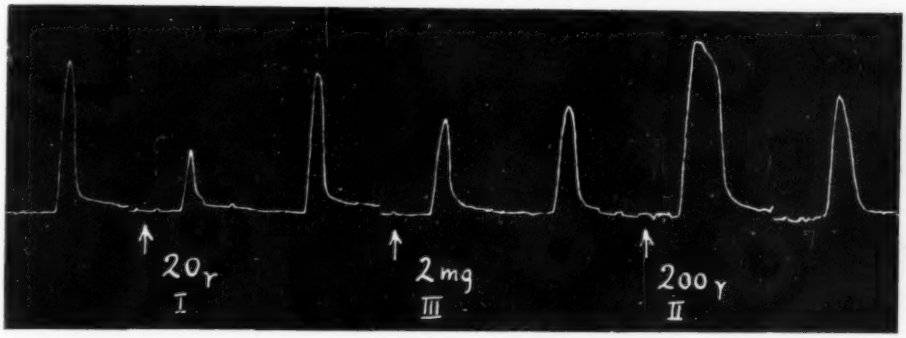


FIG. 1.—Cat, chloralose. Perfused superior cervical ganglion. Record of contractions of the nictitating membrane to intra-arterial doses of 50 μg. acetylcholine. I = F2512, III = F2559, II = F2557.

F2559, 500 μg. F2557, and 100 μg. F2512 If these doses are compared with those injected intra-arterially to depress neuromuscular transmission (Section (4)) it will be seen that they are of similar magnitude but in the reverse order, the ratio being 50 : 5 : 1.

(2) *Effect on the contractions of the nictitating membrane induced by the injection of acetylcholine into the perfused superior cervical ganglion*

Method.—The method was the same as that in Section (1).

Results.—Fig. 1 shows the effect of the three compounds given in succession. A dose as small as 20 μg. F2512 reduced the response to acetylcholine by 50 per cent ; it recovered very quickly. As much as 2 mg. F2559 were required to reduce the response by about 25 per cent, but recovery was slow. When now 200 μg. F2557 were injected the response was greatly augmented. About 400–500 μg. F2557 produced depression from which the ganglion did not recover. The ratio of paralysing doses was thus 100 : 20 : 1.

The injection of the three compounds had no action by itself except that F2512, in a dose of 50–100 μg., sometimes caused a contraction of the nictitating membrane just as *d*-tubocurarine occasionally does.

The surprising observation in these experiments was the sensitizing action of F2557 on the ganglion to the injection of acetylcholine. Probably this action is related to the inhibition of cholinesterase by this compound which will be discussed below.

(3) *Effect on the contractions of the nictitating membrane induced by the injection of acetylcholine into the denervated ganglion*

(*a*) Administration of doses into the lingual artery.

Method.—Seven to eight days after the cervical sympathetic nerve had been cut the cats were anaesthetized with chloralose. A loop was put round the external carotid artery, which was temporarily occluded while injections were made through a cannula inserted into the lingual artery and pointing backwards to the common carotid.

Results.—A slight peripheral action on the nictitating membrane itself, producing a slow small contraction, was observed after the injection of acetylcholine even after removal of the ganglion at the end of the experiment. However, the contractions of the membrane produced by the ganglionic action of acetylcholine were immediate and much larger, and the following results were obtained: No dose of F2559 up to 10 mg. depressed the response to acetylcholine. Doses of F2557 up to 5 mg. caused sensitization like that seen in Fig. 1 ; larger doses, up to 10 mg., caused some depression. The most active substance of the three was F2512, which depressed the acetylcholine response in a dose of about 100 μg. ; in this dose it was once observed to cause a contraction by itself. The ratio of paralysing doses was thus > 100 : 100 : 1.

(*b*) Experiments on the perfused denervated superior cervical ganglion.

Method.—This was the same as in (1).

Results.—Again it was found that F2559 in doses up to 10 mg. had no action, 5 mg. F2557 caused sensitization and 10 mg. depression ; 100 μg. F2512 caused a big contraction by itself (as 40 μg. tubocurarine chloride did in this same experiment) and depressed the response to acetylcholine by 50 per cent. The ratio of paralysing doses was again > 100 : 100 : 1.

Thus the difference between the responses of normal and denervated ganglia to acetylcholine was that about five times the amount of the curarizing agent was required to depress the action of acetylcholine in denervated ganglia. The activities

of the three substances remained in the same order but the ratios were very much greater.

(4) *Effect on the response of skeletal muscle to stimulation of the motor nerve*

Method.—Cats anaesthetized with chloralose were used. The contractions of the gastrocnemius to maximal single shocks (15 per min.) applied to the sciatic nerve were recorded. The compounds were injected intra-arterially through a cannula inserted into the opposite iliac artery and pointing towards the bifurcation of the aorta.

Results.—The ratio of intra-arterial doses required to reduce muscular contractions to about 50 per cent was found to be the same as that of intravenous doses. The curarizing intravenous doses were 500–750 μg. of F2559, 1–2 mg. of F2557, and 20 mg. of F2512 per kg. respectively (Depierre, 1947). By the arterial route they were 200 μg., 400 μg., and 4 mg. per kg. The ratio of curarizing doses was thus 1 : 2–4 : 20–40.

(5) *Effect on the response of skeletal muscle to close arterial injections of acetylcholine*

Method.—The tibialis anterior muscle preparation was used as described by Brown (1938) and acetylcholine was injected into the tibial artery. The compounds, however, were injected intra-

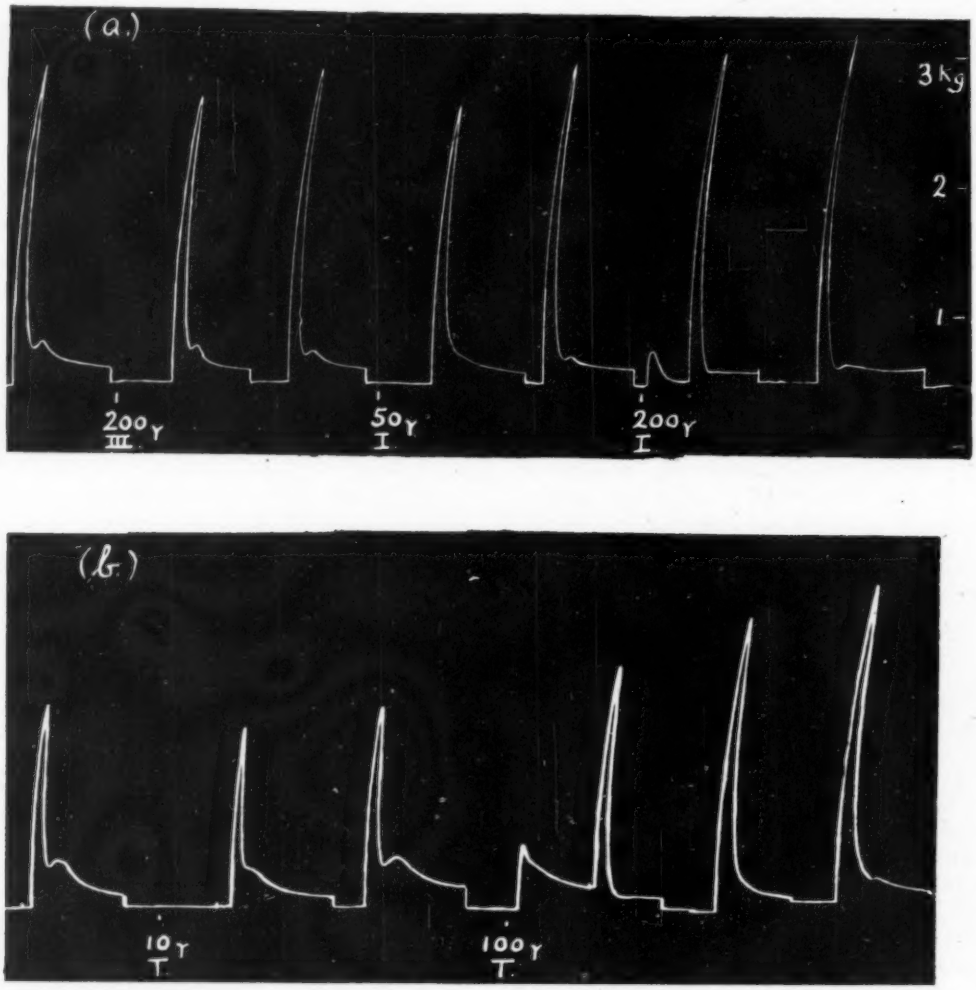


FIG. 2.—Cat, chloralose. Record of contractions of denervated gastrocnemius evoked by intra-arterial injections of acetylcholine. (*a*) Exp. No. 12, muscle denervated for 99 days, dose of acetylcholine = 30 μg. (*b*) Exp. No. 2, muscle denervated for 9 days, dose of acetylcholine = 100 μg. I = F2512, III = F2559. T = *d*-tubocurarine chloride. Note that I and T depress the response in small doses and augment it in large doses.

venously because it was found that the muscle did not recover if they were given by close arterial injection.

Results.—The doses required to produce a depression of the muscle contraction of 20 per cent were 0.4 mg. F2559, 1.5 mg. F2557, and 5 mg. F2512. For a depression of 75 per cent the doses required were 0.75 mg., 2 mg., and 100 mg. respectively. Thus the ratio was found to be about 1 : 4 : 12.

(6) *Effect on the response of denervated muscle to the distant arterial injection of acetylcholine*

Method.—The sciatic nerve was cut 6–30 days before the experiment. The cats were anaesthetized with chloralose. The contractions of the gastrocnemius were recorded, and the injections of acetylcholine, as well as of the curarizing compounds, were made into the iliac artery as in (4).

Results.—The action of the three compounds on denervated muscle was found to be very complex. F2559 was not so potent as F2512 when given in small doses, but as the dose of F2559 was increased the paralysing effect on the muscle response became greater. On the other hand, F2512 depressed the response of denervated muscle to acetylcholine in small doses only; as the dose of F2512 was increased its paralysing effect became less and less, and when given in large doses it actually increased the response to acetylcholine (Fig. 2*a*). F2557 was found to depress the muscle response to

TABLE I

PERCENTAGE CHANGES IN THE RESPONSE OF DENERVATED MUSCLE TO ACETYLCHOLINE PRODUCED BY DIFFERENT DOSES OF F2559 AND F2512

F2559

No. of exp.	Dose of ACh. μg.	20 μg.	50 μg.	100 μg.	200 μg.	400–500 μg.	1 mg.	2 mg.	4 mg.	Days denervated
2	100		−16		−40					9
3	20				−10	−17				11
4	100					+5	−50			11
5	50		−10		−20					14
6	20						−12	−10	+30	14
8	35		+8	−20						20
8	50					0	−44			20
10	20		0			−62				20
11	50			+2		−16	−19			21
12	30		+10	+5	−12	−14				29
Mean			−2	−4	−20	−17	−31	−10	+30	

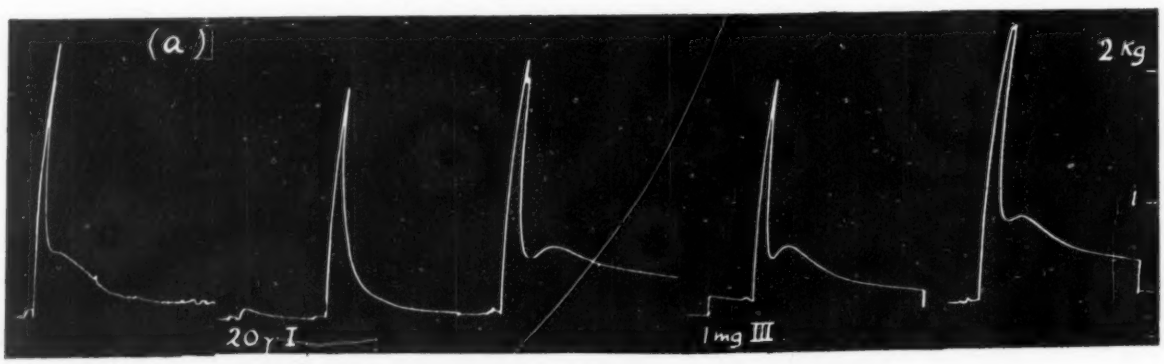
F2512

No. of exp.	Dose of ACh μg.	20 μg.	40–50 μg.	80–100 μg.	150–200 μg.	400–500 μg.	600–800 μg.	1–1.2 mg.	Days denervated
1	20		−31		−40	−14		+4	6
2	100					−10	+22		9
4	100			+16		+29			11
5	50				0			+20	14
6	20	−13							14
7	30		−35		0				15
8	35		−11	−33					20
8	50				−11			−8	20
9	50			0	−4	−28	−5	+18	20
10	20	−62	0						20
11	50			−12		+2		+10	21
12	30		−13	0	+7	+5			29
Mean		−37	−18	−6	−8	−4	+8	+9	

acetylcholine in relatively large doses only (1–2 mg.). No augmentation of the muscle response was observed (though this was regularly seen in the sympathetic ganglion), but as this compound possesses some anticholinesterase activity (see Section (8)), which would naturally interfere with its action, it was not examined so thoroughly as the other two compounds.

The results of 12 experiments in which the effects of several doses of F2559 and F2512 were compared are given in Table I. In order to make such a comparison doses of acetylcholine were given at intervals of 15 min. One minute before the injection of acetylcholine the curarizing compound was given, and care was taken not to cause too large a depression, as otherwise the previous muscle response to acetylcholine would not return; for this reason the percentage changes of the muscle response are mostly small, but in each experiment it will be seen that the depressing action of F2559 increased with increasing doses, whereas that of F2512 diminished when larger doses were used. The latter compound had a stimulating action itself; in doses of 100 μg. or more it caused a muscle contraction, and it was in this range, from 0.1 to 1.0 mg., that it often did not diminish but increased the subsequent response to acetylcholine. F2559 in doses of about 1 mg. caused a considerable depression. In one experiment only had F2559 a stimulant action itself; 1 mg. caused a small muscle contraction (see Fig. 3 (*a*)) and depressed the subsequent response to acetylcholine, whereas 4 mg. caused a large contraction and sensitized the muscle to a subsequent injection of acetylcholine (Exp. 6 in Table I).

If the percentage changes shown in Table I are taken as an index of the activities of the two compounds it will be seen that F2512 appears to have a much stronger paralysing action than F2559 in the dosage range 20–50 μg., whereas in the range 200–500 μg. this ratio of potency is completely reversed.



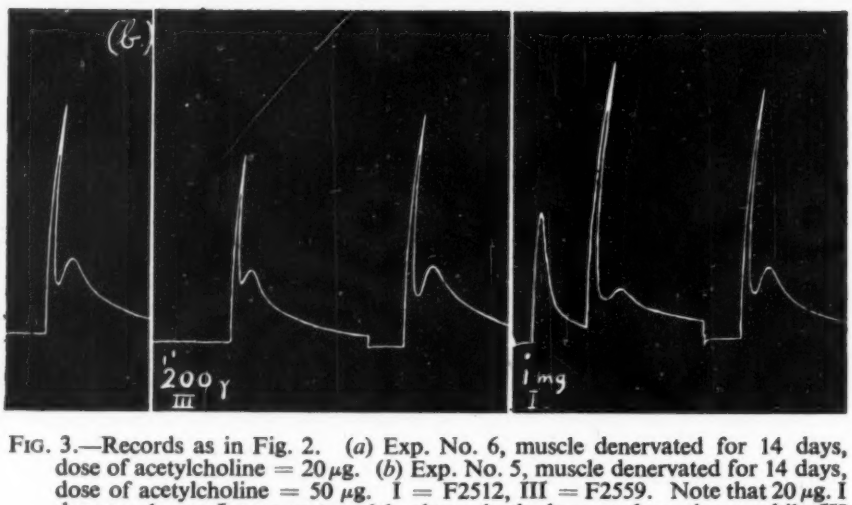


FIG. 3.—Records as in Fig. 2. (*a*) Exp. No. 6, muscle denervated for 14 days, dose of acetylcholine = 20 μg. (*b*) Exp. No. 5, muscle denervated for 14 days, dose of acetylcholine = 50 μg. I = F2512, III = F2559. Note that 20 μg. I depress, 1 mg. I augments quick phase, both depress slow phase, while III depresses quick phase only.

The difficulties encountered in assessing the relative activities of the two compounds may be seen in Fig. 2 (*a*). In this experiment 200 μg. F2559 and 50 μg. F2512 were found to be equiactive doses. However, when the dose of F2512 was increased to 200 μg. it produced not more depression but instead an augmentation of the muscle response to acetylcholine. This sensitizing action was still seen in the response to acetylcholine 15 min. later. A similar effect obtained with *d*-tubocurarine chloride is shown in Fig. 2 (*b*). A small dose, i.e., 10 μg., caused some depression (as 50 μg. F2512 in (*a*)), but a larger dose, 100 μg., not only caused a muscle contraction by itself (as 200 μg. F2512 in (*a*)) but increased the three subsequent responses to acetylcholine. It may also be noted that whereas the first quick phase of the contraction was increased the second slow phase was depressed. Again, F2512 usually, though not always, depressed the slow phase more than the quick phase, whereas F2559 depressed the quick phase and usually left the second phase unchanged. An example of this is given in Fig. 3.

The sensitizing action of F2512 was clearly related to its own stimulating action, as it mostly occurred after the compound itself had elicited a muscle contraction ; thus, in comparing its paralysing activity with that of F2559 the results obtained with small doses are the more important ones. In the experiments Nos. 1, 8, and 9 it was possible to observe a progressive depression with increasing doses and then in Nos. 1 and 9 a change to augmentation. In the remaining experiments the threshold was obviously below the smallest dose employed.

Table I also shows that the action of the two compounds was independent both of the number of days the muscle had been denervated and of the dose of acetylcholine used to elicit the muscle contraction.

(7) *Effect on the response of the isolated diaphragm to stimulation of the phrenic nerve*

Method.—The preparation was that described by Bülbring (1946) and the test was carried out as described by Chou (1947). Rats were used as well as a newborn kitten.

Results.—The ratio of potency of the three compounds on the rat's isolated muscle was different from that on the kitten's muscle, which was, however, found to be similar to that on the cat's muscle *in situ*. This species difference was first pointed out to us in a personal communication from Dr. J. W. Trevan and has recently been reported by Wien (1948). On the rat's diaphragm a 50 per cent depression of maximal muscle contractions was produced by 6–8 mg. F2559, 15–22 mg. F2557, and 3–7 mg. F2512 in a 50 cc. bath, which gives a ratio of 1 : 2 : 0.5–1 ; thus on the rat's muscle F2559 and F2512 were of about equal strength, but on the kitten's muscle F2559 was about 10 times stronger than F2512, which is a similar result to those obtained on the cat's muscle *in situ*.

(8) *Effect on cholinesterase*

Method.—The details of the method used are described by Bülbring and Chou (1947). Only the inhibition of "true" cholinesterase (dog's caudate nucleus suspension) has been studied. Mrs. P. Holton very kindly carried out these experiments for us.

Results.—It was found that F2559 and F2512 had no anti-cholinesterase activity in a concentration of $10^{-3}M$. F2557 was found to have a weak inhibitory action. The results are given in Table II. The concentration of acetylcholine used in these experiments was $6 \times 10^{-3}M$.

TABLE II

INHIBITION OF CHOLINESTERASE (DOG'S CAUDATE NUCLEUS) BY F2557

Experiment	Percentage inhibition at			
	$10^{-4}M$	$3 \times 10^{-4}M$	$10^{-3}M$	$3 \times 10^{-3}M$
1	10	—	50	—
2	7	—	47	—
3	15	26	41	64

Discussion

The compounds investigated are of special interest because they show a remarkable discrepancy between their paralysing action on neuromuscular transmission and their blocking action on synaptic transmission in the sympathetic ganglion. From the doses required to cause a given depression of the response Table III has been constructed, which gives the relative activities of the three compounds on various preparations.

The ratio of activity on denervated muscle could not be assessed, since it varied with the dose employed. Thus, if small doses of the curarizing substance, e.g., 20–100 μg., were used, F2512 was found to be 10–20 times stronger than F2559. When larger doses were given, e.g., 200 μg.–500 μg., the ratio was reversed and F2559 was found to be 2–5 times stronger than F2512. When still larger doses were used the stimulant action of F2512 itself and its sensitizing effect on the

TABLE III

Preparation	Relative activities		
	F2512	F2557	F2559
Sympathetic ganglion stimulated through preganglionic nerve	100	20	2
Sympathetic ganglion stimulated by intra-arterial acetylcholine	100	5	1
Denervated sympathetic ganglion stimulated by i.a. acetylcholine	100	1	1
Skeletal muscle stimulated through motor nerve ..	2–5	25–50	100
Skeletal muscle stimulated by intra-arterial acetylcholine ..	8	25	100

response to acetylcholine became prominent and the comparison of the two compounds became impossible. F2557, which depressed the muscle response to acetylcholine only in large doses of about 1 mg., was again intermediate in potency between the two other compounds. However, this substance has some anti-cholinesterase activity and this was probably responsible for the augmentation of the action of acetylcholine in the sympathetic ganglion. On the muscle we never observed this augmentor action, but we did not include F2557 in the detailed comparison on denervated muscle.

A striking similarity was found to exist between the inhibitory effect of F2512 and F2559 on the action of acetylcholine in the normal sympathetic ganglion and in the denervated skeletal muscle. This was evident in a certain range of doses, in which the stimulant action of the compounds themselves was too small to interfere. In the normal sympathetic ganglion the potency of F2512 was found by Depierre (1947) to be very similar to that of *d*-tubocurarine, though the effect of F2512 is short-lasting. In denervated muscle we observed the same similarity. Not only were the doses of the two substances required to depress the action of acetylcholine of the same magnitude, but *d*-tubocurarine was also found to have a stimulant action itself and to sensitize the denervated muscle to subsequent injections of acetylcholine. This observation supplements that of McIntyre and King (1943), who found that in the dog stimulant doses of *d*-tubocurarine rendered the muscle unresponsive to previously effective quantities of acetylcholine. It may be that the doses of *d*-tubocurarine used by McIntyre were larger than those we employed. We found that stimulant doses of both substances do not necessarily depress the subsequent muscle response to acetylcholine. Both *d*-tubocurarine and F2512 depress in small doses; with larger doses they sensitize; and with still larger doses they depress once more.

Summary

1. The action of β-diethylaminoethoxybenzene ethiodide (F2512), 1 : 3-bis-(β-diethylaminoethoxy)-benzene diethiodide (F2557), and 1 : 2 : 3 - tri - (β - diethylaminoethoxy)benzene triethiodide (F2559) was studied on skeletal muscle and the sympathetic ganglion, both normal and denervated.

2. On normal skeletal muscle F2559 has a strong curarizing action both when the muscle is stimulated through its nerve and when it is exposed to intra-arterial injections of acetylcholine; F2512 is 12 to 50 times weaker. This ratio of activity is reversed on the sympathetic ganglion whether it is stimulated through its preganglionic nerve or by intra-arterial injection of acetylcholine, F2512 being 50 to 100 times stronger than F2559.

3. On denervated skeletal muscle, stimulated by intra-arterial injection of acetylcholine, the ratio of activities of F2512 and F2559 is the reverse of that in normal muscle, provided that the doses of the curarizing agents are small. F2512 is then, as in the normal sympathetic ganglion, 10–20 times stronger than F2559.

4. Large doses of F2512 have a sensitizing action on denervated muscle; as the dose is increased the diminution of the muscle response to acetylcholine gives way to an augmentation. This effect has also been observed with *d*-tubocurarine.

5. F2512 and *d*-tubocurarine both have a stimulant action by themselves on the normal as well as on the denervated sympathetic ganglion and on the denervated muscle.

6. F2557 causes a 50 per cent inhibition of "true" cholinesterase (dog's caudate nucleus suspension) in a concentration $10^{-5}M$. F2512 and F2559 do not inhibit cholinesterase in this concentration.

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TUBOCURARINE ANTAGONISM AND INHIBITION OF CHOLINESTERASES

BY

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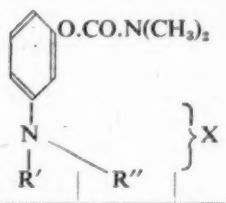
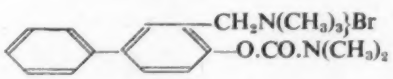
In a recent paper Bülbring and Chou (1947) compared the activities of various substances with that of prostigmine as antagonists to tubocurarine and as inhibitors of cholinesterase. The results obtained with ethyl homologues of prostigmine suggested that a parallelism might exist between the two activities, and for this reason a larger series of chemical compounds has now been investigated.

All the substances studied are given in Table I. Compounds 3392 and 3393, the mono- and di-ethyl homologues of prostigmine, are the two substances previously examined; compound S208 is the N-triethyl homologue of the same series. Compound 5220/5 is an amine oxide closely related to prostigmine; this substance is of interest as a prostigmine derivative in which the quaternary ammonium group has lost its strongly basic character. Compounds Nu 1250 and Nu 1197 are known to be strong inhibitors of true cholinesterase (Aeschlimann and Stempel, 1946), and the compound Nu 683 was included because it is

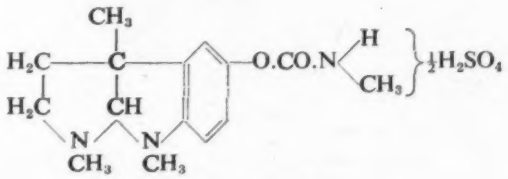
TABLE I



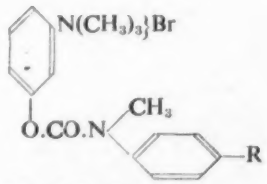
Compound	R	R′	R″	X
Prostigmine	CH_3	CH_3	CH_3	SO_4CH_3
3392 ..	CH_3	CH_3	C_2H_5	I
3393 ..	CH_3	C_2H_5	C_2H_5	I
S208 ..	C_2H_5	C_2H_5	C_2H_5	I
5220/5 ..	CH_3	CH_3	OH	Cl



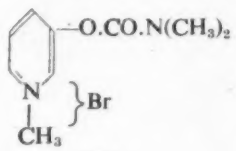
Nu 683



Eserine sulphate



Nu 1250, R = Cl
Nu 1197, R = CH_3



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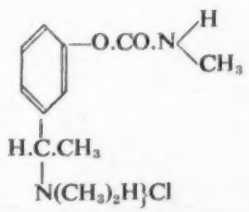
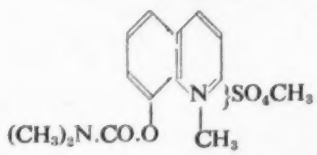


Miotine hydrochloride



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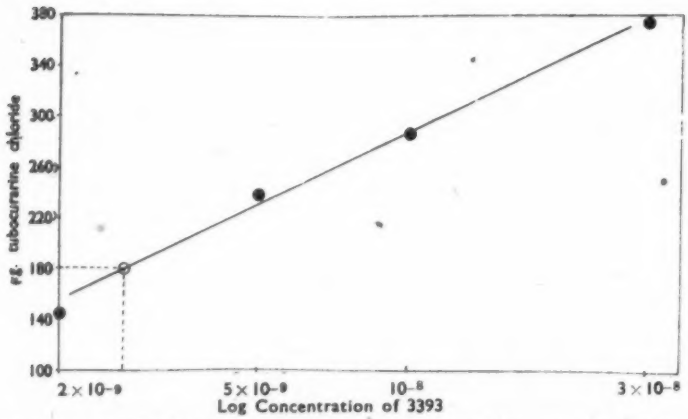

FIG. 1.—Evaluation of antitubocurarine activity on the rat's phrenic nerve-diaphragm. Ordinate = dose of *d*-tubocurarine chloride in μg. Abscissa = log concentration of 3393. The circle indicates the point from which the pD 20 value is found (see text).

known to be a specific inhibitor of pseudo-cholinesterase (Hawkins and Gunter, 1946).

METHODS

For the estimation of the anticurare activity the isolated phrenic nerve diaphragm preparation of the rat (Bülbring, 1946) was used. The quantitative evaluation was based on the fact that the dose of tubocurarine chloride required to cause a certain reduction of muscle contractions in the presence of an antagonist is proportional to the concentration of the antagonist. Bülbring and Chou (1947) expressed the potency of any antagonist in terms of that of prostigmine, which they used as a standard. For a comparison of anticurare activity with anticholinesterase activity it was, however, necessary to express the potency by an absolute rather than by a relative definition. This was done as illustrated in Fig. 1, in which the mean results of five experiments are shown. Each point represents the relation between a given concentration of 3393 (abscissae) and the dose of *d*-tubocurarine chloride (ordinates) which caused 20 per cent depression of the muscle contractions. A dose of *d*-tubocurarine chloride had to be chosen which was antagonized by most of the substances investigated within the range of concentrations used; this was 180 μg. The corresponding concentration of the antagonist was found by interpolation as shown in Fig. 1. For four compounds (38, Nu 683, 5150, and 5220/5) it had to be found by extrapolation. The potency of any tubocurarine antagonist was thus expressed as the negative logarithm of the concentration in the presence of which 180 μg. *d*-tubocurarine chloride caused 20 per cent depression of the muscle contractions. This is the pD 20 value; the figures are given in Table III.

The method of estimating anticholinesterase activity was the same as that described by Bülbring and Chou (1947). The inhibitory action on both true and pseudo-cholinesterase was studied. Each substance was therefore incubated with dog's brain (caudate nucleus) and acetylcholine (for its action on true cholinesterase) and also with horse serum and benzoylcholine (for its action on pseudocholinesterase). Table II gives the quantities of enzyme preparations and substrates used in our experiments.

RESULTS

For the estimation of anticholinesterase activity several different concentrations of the inhibitor were tested. An absolute value of inhibitory activity was obtained by finding an index which was called pI 50. Fig. 2 shows all the results obtained with different concentrations of prostigmine on the two enzymes. Each point corresponds to one observation, and the curves are drawn through the means for each prostigmine concentration. The abscissa of the point where the curve crosses the 50 per cent line is the negative logarithm of the molar concentration which causes 50 per cent inhibition: this is the pI 50 value. It can be seen in Fig. 2*a* that the pI 50 value obtained with dog's brain was 7.4. The actual figures in eight individual experiments were 7.2, 7.6, 7.5, 7.1, 7.5, 7.3, 7.3. With horse serum the individual figures in four experiments (Fig. 2*b*) were 7.2, 7.1, 7.35, 7.15, and the mean was thus 7.2. Koelle and Gilman (1946) used a similar index which they called pK. Whenever possible the pI 50 value for each enzyme preparation was determined with the inhibitor to be tested and with prostigmine on the same day.

TABLE II

Type of enzyme	Source and amount of enzyme preparation added to each flask (Total vol. = 3 c.c.)	Substrate
True cholinesterase	4 mg. dog's caudate nucleus	$6 \times 10^{-3}M$ acetylcholine
Pseudo-cholinesterase	2 c.c. horse serum	$6 \times 10^{-3}M$ benzoylcholine

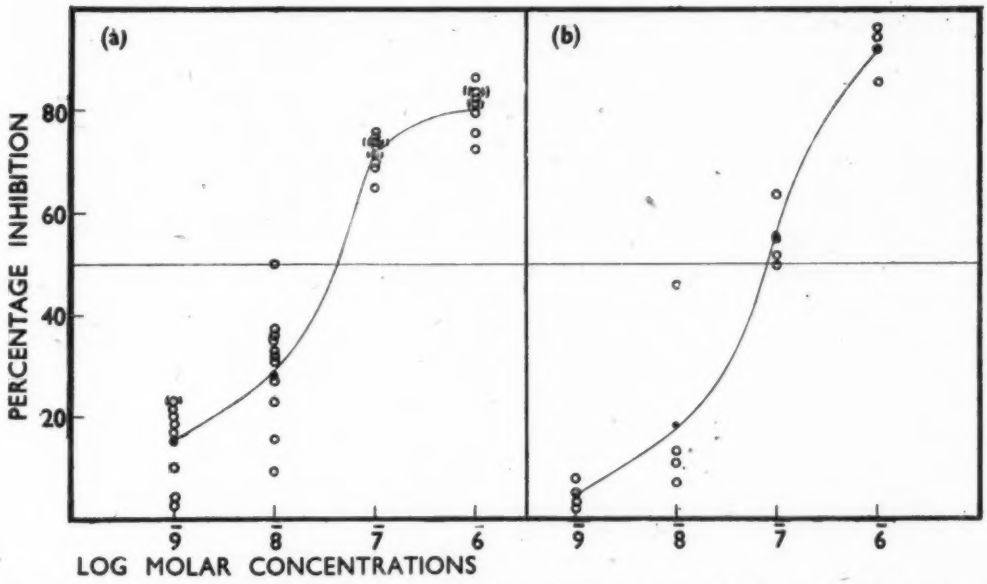

FIG. 2.—Evaluation of anticholinesterase activity of prostigmine. (*a*) Dog's caudate nucleus, (*b*) horse serum. Ordinates = percentages inhibition. Abscissae = log molar concentration of prostigmine. The pI 50 value is found from the point where the 50 per cent line crosses the curves.

The ethyl homologues of prostigmine.—Since the earlier publication of Bülbring and Chou (1947) the triethyl homologue of prostigmine (S208) has become available. It had been found previously that the substitution of one methyl group on the quaternary nitrogen by ethyl increased the activity both as inhibitor of the two cholinesterases and as antagonist to tubocurarine. The introduction of the second ethyl led to a still further increase in activity. We have now examined S208 and have found that the introduction of the third ethyl group decreases the activity in both respects below that of prostigmine itself. The figures will be found in Table III, in which all the results are summarized.

The amine-oxide homologue of prostigmine (5220/5).—This compound was without any anticurare activity in the concentration tested. The pD 20 value was determined by extrapolation and was the lowest of all. Its inhibitory action on both enzymes was also weak. It is known that the amine oxides of the general structure

TABLE III

Compounds	Mol. weight	Anticurarine activity pD 20 value	Anticholinesterase activity pI 50 value	
			On true cholinesterase	On pseudo-cholinesterase
Prostigmine ($RNMe_3$)*	334	7.60	7.4	7.2
3392 (RNMe Et)	336	8.19	8.0	7.3
3393 ($RNMeEt_2$)	350	8.57	8.2	8.0
S208 ($RNEt_3$)	392	6.59	7.2	7.4
Miotine HCl	254	7.85	7.2	6.4
Eserine sulphate	648	7.21	7.1	7.7
No. 38	354	6.23	7.1	7.6
Nu 1250	339.7	7.26	7.4	7.9
Nu 1197	379.3	7.31	6.9	7.1
Nu 683	388	5.44	6.2	8.5
5130	251	5.26	6.4	5.8
5220/5 ($RNMe_2OH$)	240.5	4.77	4.5	4.4

*R = m-$(CH_3)_3N.CO_2C_6H_4$-

R,NOH} OH (where R is an alkyl or aromatic radical) are bases with very small dissociation constants and in aqueous solution they exist almost entirely as undissociated molecules. For instance, the salts of trimethylamine oxide, Me,NOH}I, show an acid reaction in solution. Our results therefore reveal that the strongly basic character of the phenolic nitrogen radical is indispensable for a substance with strong anticholinesterase and anticurare activity.

Substances with predominantly inhibitor action on pseudo-cholinesterase.—Hawkins and Gunter (1946) found that the substance Nu 683 was a strong inhibitor of pseudo-cholinesterase, whereas it affected true cholinesterase only in very high concentrations. In the living animal it produced symptoms of acetylcholine poisoning only in doses which significantly reduced the action of the true enzyme. The authors concluded that these results indicated that pseudo-cholinesterase played no essential part in the hydrolysis of acetylcholine *in vivo*. We found that both the anticurare action of Nu 683 and its inhibitory action on true cholinesterase were low, whereas its action on pseudo-cholinesterase was high.

The parallelism between anticurarine activity and inhibitory action on true cholinesterase.—Inspection of Table III will show that the different substances do not always have the same inhibitory action on both esterases; some inhibit pseudo-cholinesterase more strongly than true cholinesterase and vice versa. On the whole, however, there is good agreement between the degree of inhibitory activity on true cholinesterase and that of antitubocurarine activity, whereas no such correlation exists between the action on pseudo-cholinesterase and tubocurarine antagonism. We are very grateful to Dr. W. Perry for the statistical analysis of the results in Table III, by which it was established that a significant correlation exists between the values for anticurare activity and those for anti-true-cholinesterase activity ($P<0.001$), whereas there is no indication of a similar correlation between anticurare and anti-pseudo-cholinesterase activities ($P>0.1$).

Discussion

Our results show that there is a significant correlation between antitubocurarine activity and inhibitory action on true cholinesterase. This supports the view of Hawkins and Mendel (1947) that it is the true cholinesterase which is responsible for the destruction of acetylcholine at the site of its physiological action.

Aeschlimann and Stempel (1946) have estimated the inhibition of a purified specific cholinesterase preparation from the electric eel by Nu 1250 and Nu 1197. They found that the anticholinesterase activity of Nu 1250 was five times, and that of Nu 1197 more than equal to, that of prostigmine. We find that the first substance has the same activity as prostigmine, and the second substance has less activity. A possible reason for this discrepancy is the different source of the enzyme; we have used a suspension of the dog's caudate nucleus and consequently our tests both for anticurare activity and for anticholinesterase activity were carried out with mammalian tissues.

It is known that structural changes of the prostigmine molecule sometimes increase the affinity for one enzyme and decrease it for the other. One example is Nu 683, which is of special interest here as the decline in inhibitory action on true cholinesterase finds its parallel in weaker anticurare activity.

An important result of our experiments is the finding that a strongly basic nitrogen radical in the phenolic moiety of the molecule is indispensable not only for a strong anticurare activity but also for a strong anticholinesterase activity.

The ethyl homologues of prostigmine provide an interesting group of compounds: substitution of the methyl groups on the quaternary nitrogen of prostigmine by ethyl at first increases both the antitubocurarine and the anticholinesterase activity, but the peak is reached at the diethyl compound and the activity of the triethyl compound is again much less.

Summary

1. The antitubocurarine activity of a series of compounds related to prostigmine was compared with their inhibitory action on the true cholinesterase of dog's caudate nucleus and on the pseudo-cholinesterase of horse serum.

2. Significant correlation was found between the inhibition of true cholinesterase and the antagonism to *d*-tubocurarine.

Thanks are due to Dr. J. A. Aeschlimann and Dr. F. Bergel for some of the substances used.

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THE ACTION OF LOCAL ANAESTHETICS AND *d*-TUBOCURARINE ON THE ISOLATED INTESTINE OF THE RABBIT AND GUINEA-PIG

BY

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This paper describes the action of local anaesthetics and of *d*-tubocurarine chloride on isolated intestinal preparations. In order to distinguish experimentally between the action of these substances on the muscle fibres and their action on the nervous structures present in the preparation the following procedure was adopted: an effect on the tone and spontaneous rhythm of the muscle and on the responses to acetylcholine or histamine was taken as evidence for action on the excitability of the muscle fibres; on the other hand the nicotine response and the local peristaltic reflex provided reactions with which the excitability of the nervous structures in the intestine could be tested.

METHODS

The experiments were carried out on isolated preparations of the rabbit's and guinea-pig's ileum suspended in aerated Tyrode's solution. A 16 c.c. bath was used when the contractions of the longitudinal muscle alone were recorded; this was done with a Lovatt Evans frontal writing lever. In the experiments on the peristaltic reflex the intestinal volume was recorded according to the method described by Trendelenburg (1917). A piston recorder of the Brodie type was used for this purpose, and the bath volume was 35 c.c. The method of suspending the intestinal preparation was identical with that described by Feldberg and Solandt (1942). The temperature of the bath was kept at 37° C. when the rabbit's, and at 35° C. when the guinea-pig's, ileum was used. The animals were killed by a blow on the head immediately before the intestine was removed.

The local anaesthetics used were cocaine hydrochloride, procaine hydrochloride, and nupercaine hydrochloride. The amounts stated in the text refer to the salts and not to the bases.

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†British Council Fellow.

RESULTS

I. Effect on the excitability of the muscle fibres

Movements and tone of the muscles of the small intestine of most animals are increased by small doses of cocaine and decreased by large ones (von Anrep, 1880; Bayliss and Starling, 1899; Langley and Magnus, 1905; Kuroda, 1915; and Trendelenburg, 1917). On the guinea-pig's intestine, however, the action of cocaine was found by Trendelenburg to be always depressant. In our experience this was not so.

Guinea-pig.—In some preparations small doses of cocaine had a stimulating effect. The contractions of the longitudinal muscle were usually found to be small and transient and obtainable only when cocaine was added to the bath for the first time. In a few preparations, however, cocaine produced strong and well-maintained contractions which could be obtained with each new administration of the drug and resembled the contractions produced by acetylcholine or histamine. Even when cocaine had no stimulating action of its own, small doses often rendered the preparation more sensitive to the stimulating action of histamine or acetylcholine.

In order to obtain the depressant action of cocaine it had to be given in concentrations stronger than 1 in 80,000 (see Fig. 1*b*). There might then be some relaxation of the tone of the longitudinal muscle and reduction of the spontaneous contractions if they were present. The most obvious effect, however, was the reduction in the response to acetylcholine, histamine, or potassium. The depression was greatest during the first few minutes of the cocaine action and decreased somewhat when the cocaine was kept in the bath for longer periods. After the cocaine had been

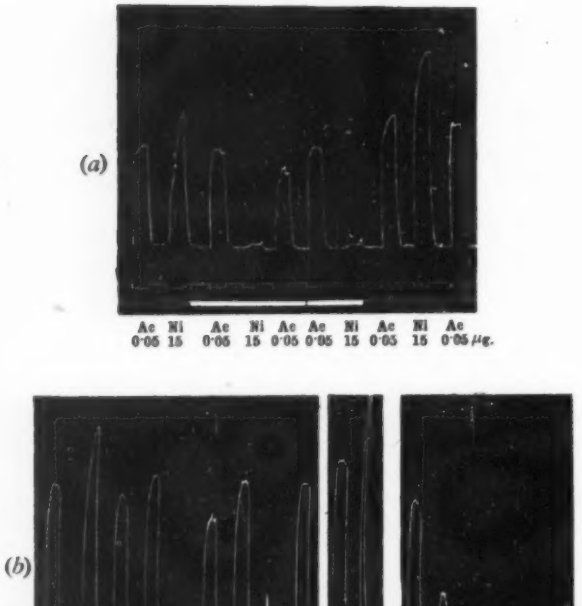


FIG. 1.—Contractions of guinea-pig's ileum in 16 c.c. Tyrode's solution. At (Ac) acetylcholine chloride, at (Ni) nicotine tartrate in μg., as indicated at the bottom of the figure, added to the bath. The broad white lines indicate the periods during which the bath contained cocaine, 1 in 80,000 (first two white lines) and 1 in 25,000 (last white line).

washed out the muscle quickly regained its original sensitivity, in fact for 10 to 15 min. it became even more sensitive to histamine or acetylcholine than it had been before the cocaine administration (see Fig. 1).

During a cocaine depression the muscle was unable to contract as strongly as the untreated muscle even if acetylcholine or histamine were given in hundredfold increased amounts. The extent of the reduction in contractibility depended on the concentration of cocaine present in the bath, becoming more pronounced with increasing concentration.

Other local anaesthetics examined depressed the muscle fibres in weaker concentrations than cocaine. A reduction of the acetylcholine response similar to that seen with 1 in 35,000 cocaine was seen with concentrations of 1 in 600,000 procaine and 1 in 800,000 nupercaine.

d-Tubocurarine chloride, even if given in strong concentrations (1 in 80,000 to 1 in 16,000), did not affect the muscle tone and had only a slight and irregular effect on the responses to acetylcholine and to histamine. The responses to histamine were sometimes slightly depressed, sometimes slightly augmented; those to acetylcholine, however, were usually slightly depressed; the depression did not become more pronounced when the concentration of tubocurarine was increased. After a strong concentration of tubocurarine had been washed out there was often a period of a few minutes in which the response to acetylcholine was augmented.

Rabbit.—In some preparations cocaine increased, in others it decreased, the amplitude of the rhythmic contractions and caused slight loss of tone of the longitudinal muscle. When different parts of the intestine were examined inhibition by cocaine was found to occur more regularly in the duodenum and jejunum and stimulation in the ileum, so that sometimes cocaine had opposite effects in different parts of the same intestine. In order to obtain inhibition of the acetylcholine response cocaine had to be given in concentrations higher than 1 in 30,000, except in preparations which had been kept suspended in the bath for several hours and had become more sensitive to the depressant action of cocaine. Nupercaine was about 20 times as active as cocaine. A similar inhibitory action of other local anaesthetics on the rabbit's duodenum has been described by Tripod (1940), and more recently by Dawes (1946) and de Elío (1948).

d-Tubocurarine chloride in a concentration of 1 in 30,000 or stronger usually caused slight and transient augmentation of the tone and amplitude of the rhythmic contractions. Since this effect was not prevented by the presence of a strong concentration of local anaesthetics in the bath fluid, it could not have been due to stimulation of the ganglion cells in the intestinal wall. In some preparations weak concentrations of tubocurarine (about 1 in 200,000) had a slight depressant effect on the response to acetylcholine, in others such an effect was obtained with very strong concentrations only (1 in 40,000 to 1 in 16,000).

II. Effect on the nervous structures in the intestinal preparation

(a) The nicotine response of the guinea-pig's ileum

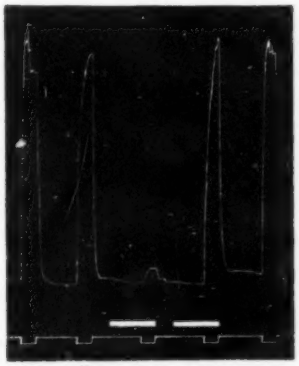
The addition of small doses of nicotine to the bath in which a guinea-pig's ileum is suspended produces contractions which differ from those produced by acetylcholine or histamine in that the

onset is delayed for a few seconds and the contraction itself proceeds more gradually. If a few minutes are allowed each time after the nicotine has been washed out and before its renewed administration, comparable results can be obtained without manifestations of a paralysing action of nicotine, but when such an experiment is continued for long periods the responses to nicotine often become gradually smaller.

The nicotine contractions are abolished by concentrations of local anaesthetics or tubocurarine chloride which do not yet affect or affect slightly only equally strong contractions produced by histamine or acetylcholine. In the experiment of Fig. 1, doses of 15 and 30 μg. of nicotine had stronger actions than 0.05 and 0.1 μg. of acetylcholine respectively. Cocaine (1 in 80,000) prevented the nicotine responses but left those to acetylcholine practically unaffected. The preparation was not completely insensitive to nicotine, which in a dose of 200 μg. caused a small contraction. In order to abolish this response the concentration of cocaine had to be increased to such an extent that it reduced the response to acetylcholine, i.e., the excitability of the muscle fibres (Fig. 1*b*). An experiment like that for cocaine in Fig. 1*a* is shown for tubocurarine in Fig. 2.

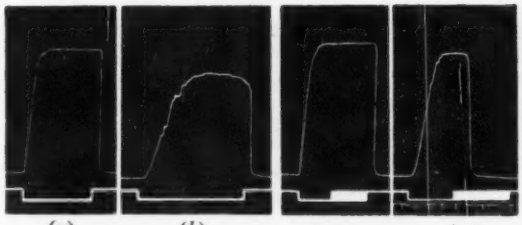
In the experiment of Fig. 3 the procedure was slightly modified. It illustrates that an acetylcholine, but not a nicotine, contraction is maintained in the presence of 1 in 80,000 cocaine; relaxation of the nicotine contraction started 13 sec. after adding the cocaine to the bath.

The inhibiting effect of cocaine and tubocurarine on the nicotine response is not so strong as that produced by paralysing doses of nicotine itself.



(*a*) (*b*) (*c*) (*d*) (*e*)

FIG. 2.—Contractions of guinea-pig's ileum in 16 c.c. Tyrode's solution. At (*a*), (*c*), and (*e*) 40 μg. nicotine tartrate, at (*b*) and (*d*) 0.4 μg. acetylcholine chloride for 1 min. The broad white lines indicate the presence of 200 μg. *d*-tubocurarine chloride in bath.



(*a*) (*b*) (*c*) (*d*)

FIG. 3.—Contractions of guinea-pig's ileum in 16 c.c. Tyrode s solution. At (*a*) and (*c*) 0.4 μg. acetylcholine chloride kept in the bath for 2½ min., but followed after the 1st min. at (*c*) by 200 μg. cocaine. At (*b*) and (*d*) 35 μg. nicotine tartrate kept in the bath for 4 min., but followed after the 2nd min. at (*d*) by 200 μg. cocaine. Period during which cocaine was present in bath indicated by broad white line. Time in 30 sec.

In the presence of 1 in 200,000 nicotine the preparation is paralysed to any dose of nicotine; but 1 in 40,000 tubocurarine chloride renders the preparation insensitive to small doses only of nicotine, the effect of 100 or 200 μg. of nicotine being greatly reduced but not abolished. Usually the sensitivity of the preparations to the inhibitory action of cocaine and tubocurarine increased after a few hours' suspension in the bath of Tyrode's solution.

(b) The peristaltic reflex in the rabbit's ileum

The reflex was initiated by raising the pressure in the lumen of the intestine by 2.5 to 4 centimetres of saline. In some preparations, in which the increased pressure in the lumen was maintained, peristaltic waves continued for hours at regular intervals of 15 to 25 sec., interrupted from time to time by short periods of inactivity of the circular muscle. In other preparations cessation of the reflex took place within a few minutes after the pressure in the lumen had been raised. The procedure usually adopted was to raise the pressure at every 4th min. for 1 min., and to keep it at zero or even below zero during the rest of the time. The reflex could then usually be initiated for hours without signs of fatigue, but in a few experiments the peristaltic reflex was not maintained even for the 1-min. period and in others fatigue occurred in the course of a prolonged experiment. Some improvement was obtained by adding 1 in 100,000,000 to 200,000,000 eserine to the Tyrode's solution and by keeping the pressure in the lumen of the intestine at zero for 60 to 90 min. before starting or continuing the experiment. This procedure was adopted as a routine in all experiments in which it was not possible to obtain the peristaltic reflex regularly. But even this procedure proved ineffective in a few preparations, which therefore had to be discarded.

As stated by Trendelenburg (1917) the onset of the contraction of the circular muscle layer; which starts at the stomach and spreads to the caecal end, is preceded by shortening of the preparation owing to contraction of the longitudinal muscle fibres. The degree of this shortening is dependent on the tone of the longitudinal muscle and varies greatly in different preparations. In addition we found that slight variations in the initial pressure in the lumen of the intestine greatly influenced the result. With a negative pressure in the lumen the longitudinal muscle is well relaxed and, when the pressure is raised, it contracts strongly before the first wave of contraction of the circular muscle spreads over the preparation. With an initial slight positive pressure in the lumen, the longitudinal muscle is already partly contracted and the additional rise in pressure leads to a relatively small further shortening of the preparation before it is stopped by the onset of the contraction of the circular muscle layer. In Fig. 4*a* and *c* the differences in the degrees of shortening in two preparations are illustrated; the initial pressure in the lumen at *a* was about zero, at *c* −1 cm. saline.

The fact that it is really the activity of the circular muscle which interupts the contraction of the longitudinal one can be seen from the results obtained when the pressure in the lumen was lowered at different phases of the peristaltic reflex. When this occurred during the end of a wave of contraction of the circular muscle there was no further shortening, but when it occurred in the interval between two peristaltic waves, when the longitudinal muscle had begun to contract, the shortening continued for some time after the pressure had been lowered. These differences are illustrated in the two controls shown in Fig. 7, before the administration of nupercaine.

With a negative pressure of 2 or 3 cm. saline in the lumen of the intestine its walls are in apposition, the longitudinal muscle is well relaxed and exhibits no or only small rhythmic contractions.

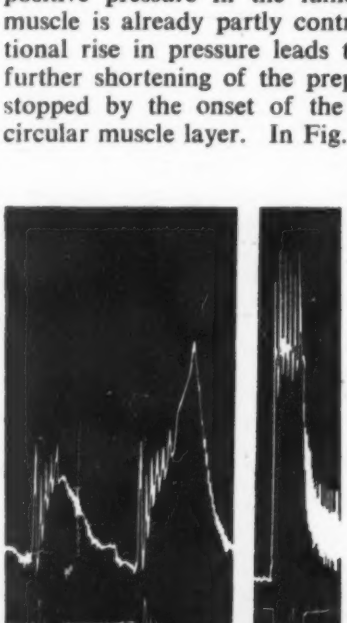


FIG. 4

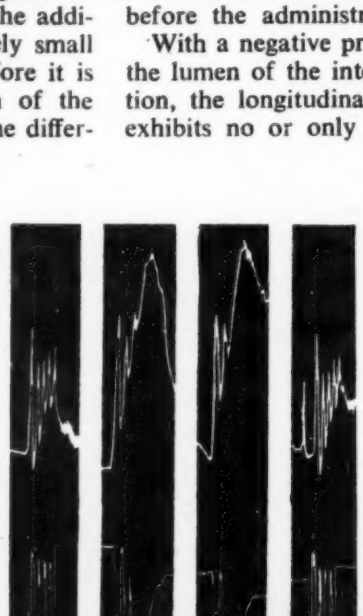


FIG. 5

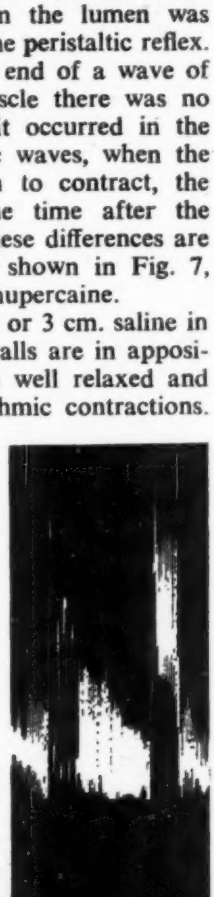


FIG. 6

FIG. 4.—Rabbit's ileum in 35 c.c. Tyrode's solution (6 μg./l. eserine). In this and the following figures the upper tracing records contractions of the longitudinal muscle, the lower tracing the intestinal volume. At (*a*), (*b*), and (*c*) pressure raised in intestinal lumen to about 3 cm. saline during the periods indicated. Time in 30 sec. Details in text.

FIG. 5.—Rabbit's ileum in 35 c.c. Tyrode's solution (6 μg./l. eserine). Effect of temperature on peristaltic reflex initiated by raising pressure in intestinal lumen to about 3 cm. saline for 1 min. Time in 30 sec.

FIG. 6.—Rabbit's ileum in 35 c.c. Tyrode's solution (6 μg./l. eserine). Effect of cocaine on peristaltic reflex. Pressure raised in intestinal lumen to about 3 cm. saline for 1 min. at (*a*) and (*b*). Broad white line indicates presence of 100 μg. cocaine in the bath. Time in 30 sec. Details in text.

In this condition the preparation is particularly suitable for testing quantitatively the effects of drugs like acetylcholine on the longitudinal muscle. The first effect of a rise of pressure in the lumen is the reappearance, or an increase in the amplitude, of the spontaneous rhythmic contractions; with a further rise in pressure the tone of the longitudinal muscle increases.

When fatigue of the peristaltic reflex takes place the response of the longitudinal muscle is not affected. The muscle either remains shortened or continues to do so after the contractions of the circular muscle have ceased (see Fig. 4*b*), and if the preparation had shown strong rhythmic contractions before the initiation of the reflex they reappear. The tracings then obtained resemble that illustrated for cocaine in Fig. 6.

The effect of lowering the bath temperature is seen in the experiment of Fig. 5. At 37° C. a 1-min. period of increased pressure in the intestinal lumen caused four peristaltic waves to spread over the whole length of the preparation (*a* and *d*); at 33.5° C. there were two such waves and a third incomplete one (*b*), and at 32.5° C. there occurred one full and one incomplete wave only (*c*). The contraction of the longitudinal muscle, however, was not inhibited when the bath temperature was lowered and the activity of the circular muscle had come to an end.

Local anaesthetics.—Von Anrep in 1880 and Bayliss and Starling in 1889 described inhibition of the peristaltic reflex in the dog when cocaine was applied in strong concentrations to the serosa of the small intestine *in situ*. According to Bayliss and Starling the spontaneous movements increased at the same time. On the isolated small intestine preparations of the rabbit and dog Trendelenburg (1917) obtained increased peristalsis with weak, but inhibition with strong, concentrations.

In our experiments the main effect of cocaine was inhibition of the peristaltic reflex, although there was some indication of a stimulating action. The inhibition by cocaine resembled the effect of fatigue or of lowering the bath temperature—i.e., the contractions of the circular but not of the longitudinal muscle were affected (see Figs. 6, 7, and 8). The concentrations of local anaesthetics necessary to inhibit the peristaltic reflex were of the same order as those which inhibit the response to small doses of nicotine.

In the experiment of Fig. 6 raising the pressure in the lumen of the intestine to 3 cm. saline for 1 min. produced four waves of contraction of the circular muscle layer (at *a*). At *b* 2 min. after the addition of 100 μg. of cocaine to the 35 c.c. bath these contractions no longer occurred when the pressure was raised, but the longitudinal muscle shortened and, in addition, exhibited its strong rhythmic contractions. The Figure illustrates well the difference in the mechanisms responsible for the contractions of the two muscle layers when the pressure in the lumen is raised. The waves of contraction of the circular muscle are inhibited because a local nerve reflex is involved in their initiation; the spontaneous rhythmic contractions, the so-called pendular movements, and the increase in tone of the longitudinal muscle persist because they are myogenic in origin. In the experiment of Fig. 7 raising the pressure in the lumen after the addition of 100 μg. cocaine to the bath still produced an initial small contraction of the circular muscle which affected the upper end of the preparation only; the longitudinal muscle, on the other hand, continued to contract during the whole period of increased pressure The quick reversi-

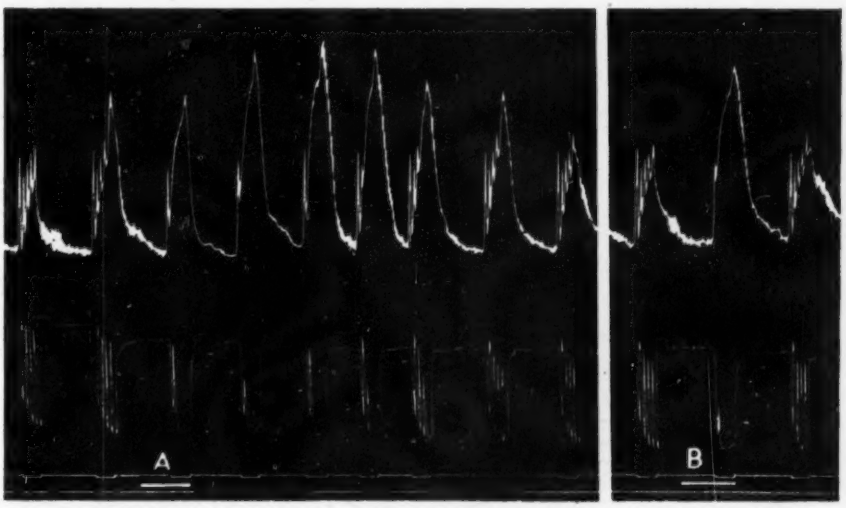


FIG. 7.—Rabbit's ileum in 35 c.c. Tyrode's solution (6 μg./l. eserin). Comparison of 8 μg. nupercaine (A) and 100 μg. cocaine (B) on peristaltic reflex. Pressure in intestinal lumen raised each time to about 3 cm. saline for 1 min. Broad white line indicates presence of local anaesthetics in bath. Time in 30 sec.

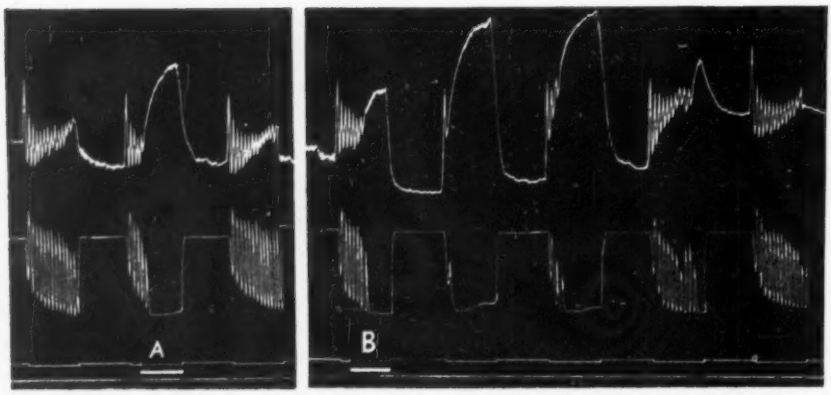


FIG. 8.—Continuation of Fig. 6. Pressure in intestinal lumen raised for 3 min. each time. Comparison of 100 μg. cocaine (A) and 10 μg. nupercaine (B) given 30 sec. after initiation of peristaltic reflex. Broad white lines indicate presence of local anaesthetics in bath. Time in 30 sec.

bility of the action of cocaine is seen from the fact that 3 min. after the cocaine had been washed out the peristaltic reflex was restored; indeed, it often became more regular and any signs of fatigue originally present disappeared. For instance, in the experiment of Fig. 8A the last two waves of contraction of the circular muscle occurring during the first 3-min. period of increased pressure in the lumen of the intestine were incomplete. After the cocaine had been washed out this "fatigue" was no longer seen. We do not know if this effect of cocaine is the result of increased excitability of the muscle fibres, or of the nervous elements, or of both.

The inhibitory effect of cocaine develops quickly. In the experiment of Fig. 8A the addition of cocaine to the bath 30 sec. after the peristaltic reflex had been initiated caused cessation of the peristaltic waves within 40 sec.

Partial inhibition of the peristaltic reflex which occurs with small doses of cocaine shows the following characteristics: (i) the contractions of the circular muscle layer come to an end after a short time; instead of the four or five waves usually passing over the preparation during a 1-min. period of increased pressure there remain the initial two or three waves or even a single contraction only; (ii) the waves of contraction become incomplete and confined to the upper end; or (iii) they become irregular and start at the middle or lower end or at different points simultaneously; dilated and constricted segments then alternate with each other. Such disorderly contractions are also frequently seen during the recovery period after a dose of cocaine greater than that necessary to produce complete inhibition of the peristaltic waves.

When cocaine was used in concentrations so strong as to depress the excitability of the muscle fibres the response of the longitudinal muscle to the stimulus of increased pressure in the lumen was also abolished.

The fact that the inhibiting action of cocaine on the peristaltic reflex is easily reversible, so that it is possible to obtain comparable effects on repeated administration of the same dose and graded responses with different doses, makes it possible to employ this reaction for the biological assay of cocaine and to compare its action with that of other local anaesthetics. When this was done it was found that procaine was slightly less active than cocaine in inhibiting the peristaltic reflex, whereas nupercaine was 12 to 12½ times as active as cocaine and, in addition, the effect of nupercaine was more prolonged and developed more gradually. In the experiment of Fig. 7 the nupercaine was added to the bath 2 min. before the pressure in the lumen was raised for 1 min. and washed out after 3 min. This was the procedure usually adopted when assaying the potency of local anaesthetics against cocaine. The greatest inhibition of the reflex did not occur during the presence of nupercaine in the bath but 3 min. after it had been washed out, and about 20 min. elapsed before the reflex was restored. In the experiment of Fig. 8, 8 μg. nupercaine and 100 μg. cocaine were added to the bath after the reflex had begun. With cocaine it took about 40, with nupercaine about 70, sec. before the reflex was inhibited, and again the prolonged effect of nupercaine was evident. This delay in the action of nupercaine was taken into account when assaying its potency against cocaine. The maximal inhibition observed after the nupercaine had been washed out was used for comparison.

d-*Tubocurarine chloride*.—This drug has only an inhibitory and no stimulating action on the peristaltic reflex. As with the inhibitory effect of cocaine only the waves of contractions of the circular muscle layer are abolished, whereas the rhythmic contractions of the longitudinal muscle, if present, and its tonic contraction in response to the increase in pressure in the lumen, persist; this is illustrated in Fig. 9. In sensitive preparations

some inhibition of the reflex is seen with concentrations less than 1 in 10,000,000 (Fig. 10), but for complete inhibition higher concentrations are necessary. The effect is easily reversible; recovery is delayed for more than a few minutes only when concentrations are used which are stronger than those necessary for complete inhibition of the reflex. Graded responses are obtained with different doses and comparable results with the same dose given repeatedly.

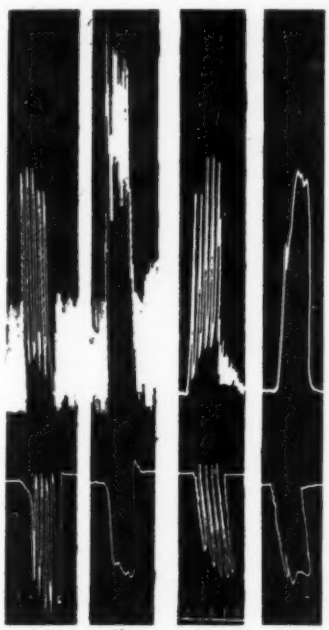


FIG. 9.—Rabbit's ileum in 35 c.c. Tyrode's solution (6 μg./l. eserine). The tracings (*a*) and (*b*) are from one, the tracings (*c*) and (*d*) from another, intestine. Inhibition of peristaltic reflex by 100 μg. *d*-tubocurarine chloride at (*b*) and (*d*), given 2 min. before the initiation of the reflex. Pressure in intestinal lumen raised each time for 1 min. to about 3 cm. saline. Time in 30 sec.

The inhibition of the peristaltic reflex can be used as a reaction for assaying unknown solutions of tubocurarine and other substances with curare-like action. Fig. 10 illustrates the inhibition produced by different doses of *d*-tubocurarine chloride. With 100 and 50 μg. inhibition was complete; with 25 μg. one wave, with 10 μg. one and a half, with 5 μg. practically two, and with 2 μg. two and a half waves remained. Each dose of tubocurarine was added to the bath 2 min. before the pressure was raised and washed out when the pressure was lowered again. The reflex was tested every 4th min. After the larger doses of tubocurarine recovery was delayed; the reflex had not fully returned when it was tested 3 and 7 min. after the tubocurarine had been washed out; it was retested only after full recovery had taken place, but with the exception of four controls, marked 0, all intermediate tests have been omitted in the Figure.

In the experiment of Fig. 10 slight quick volume changes of the intestine occurred during the tubocurarine paralysis; their frequency was the same as that of the rhythmic contractions of the longitudinal muscle and they might in part have been accounted for in this way. Partly, however, they resulted from the fact that the circular muscle layer itself exhibited rhythmic contractions at the lower end of the preparation; they were apparently myogenic in origin.

(*c*) *The peristaltic reflex of the guinea-pig's ileum*

The reflex could be obtained regularly, repeatedly, and without the necessity of adding eserine to the bath fluid. A rise of pressure of 2 cm. of saline in the lumen of the intestine was sufficient when the preparation was from a small, and of 2.5 cm. when from a large, guinea-pig. During a 1-min. period of increased pressure in the lumen, between 9 and 14 waves of contraction of the circular muscle layer passed over the preparation, and if the increased pressure was repeated every 4th min. the preparation remained active for many hours, sometimes up to 10 hours.

According to Trendelenburg cocaine had no stimulating effect on the peristaltic reflex of the guinea-pig's intestine: when kept in the bath for 1 min. cocaine had no effect at all, when kept for 5 min. the reflex was abolished. Our results were slightly different.

Usually no definite inhibitory effect on the peristaltic reflex could be obtained with cocaine, nupercaine, or tubocurarine during the first hour or so of an experiment, even if the substances were tested in relatively high concentrations and kept in the bath for several minutes. But nearly all preparations gradually became more sensitive to the inhibiting action of these substances, and then inhibition occurred whether the substances were kept in the bath for 5 min. or for 1 min. only.

During the first hour or so of an experiment the characteristic effect of cocaine, and to a lesser degree of nupercaine and tubocurarine, consisted in an increase in the frequency of the waves of contraction of the circular muscle layer. In some preparations in which 1 in 40,000 cocaine had this effect at the beginning of the experiment a 10 times weaker concentration caused complete inhibition

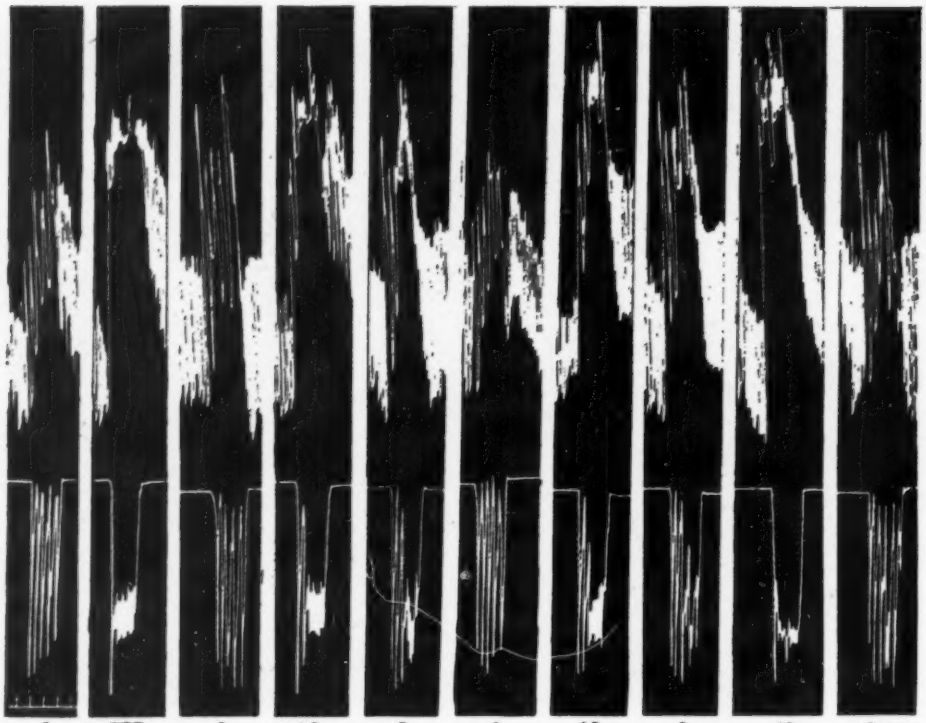


FIG. 10.—Rabbit's ileum in 35 c.c. Tyrode's solution (6 μg./l. eserine). Graded inhibition of peristaltic reflex by varying amounts of *d*-tubocurarine chloride. Amounts indicated in μg. at bottom of figure. Time in 30 sec. Details in text.

after the intestine had been in the bath for several hours. Although Trendelenburg does not mention this initial action of cocaine on the frequency of the peristaltic waves it is evident in an experiment which he illustrates in his paper.

In the experiments of Fig. 11 the different actions of cocaine on the peristaltic reflex are illustrated. Instead of the 12 peristaltic waves elicited by a 1-min. period of increased pressure in the lumen at *a*, there were 20 waves when the procedure was repeated at *b* 2 min. after the addition of 100 μg. of cocaine to the 35 c.c. bath. Sometimes up to 30 waves occurred. Each of these waves started at the upper end and extended over the whole preparation. This was not always so; often they were incomplete, irregular, and started at different parts of the preparation; at the same time the longitudinal muscle contracted. Such a result can be seen in the experiment of Fig. 11 *c* to *f*, in which another intestine was used. Instead of the 11 complete waves at *c* there were 18 incomplete waves along with shortening of the preparation at *d*. At a later stage of the experiment cocaine started to exert its inhibiting action and results like that seen at *e* were obtained: an initial period of incomplete waves at a high frequency was followed by cessation of the reflex. At an even later stage of the experiment there was complete inhibition of the peristaltic waves after the addition of the same dose of cocaine. When it was washed out and the peristaltic reflex retested at 4-min. intervals restoration of the reflex passed through a stage in which the frequency of the peristaltic waves was increased.

It was found difficult and sometimes impossible to obtain graded responses with increasing doses of cocaine and equal responses with the same dose, even at a later stage of an experiment, when cocaine exerted its strong inhibiting action on the peristaltic reflex. The isolated intestine of the guinea-pig, unlike that of the rabbit, therefore, is unsuitable for assaying the potency of an unknown solution of cocaine.

With nupercaine there were changes in the sensitivity of the preparation similar to those with cocaine, but it was usually possible to obtain some

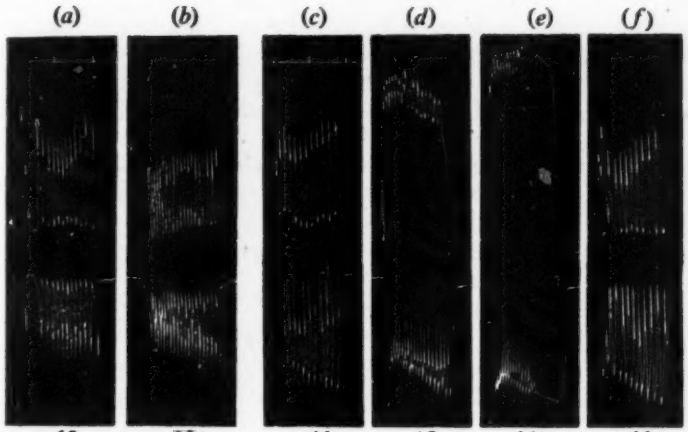


FIG. 11.—Guinea-pig's ileum in 35 c.c. Tyrode's solution. Effects of 100 μg. (at *b*) and 300 μg. (at *d* and *e*) of cocaine on the peristaltic reflex. The number of peristaltic waves during each 1-min. period is given at the bottom. Time in 30 sec. Details in text.

slight inhibition of the reflex even at the beginning of an experiment; therefore, when the potency of nupercaine was compared with that of cocaine no constant results could be obtained. In one preparation, for instance, 20 μg. nupercaine produced slight inhibition of the reflex at the beginning of the experiment whilst 800 μg. cocaine only increased the frequency of the peristaltic waves; 4 hours later 100 μg. cocaine as well as 20 μg. nupercaine abolished the reflex. In this experiment, therefore, nupercaine had an inhibiting action on the peristaltic reflex 40 times stronger than cocaine at the beginning of the experiment, but later on it was only five times as active.

The increase in the frequency of the peristaltic waves produced by nupercaine was not so pronounced as with cocaine. When nupercaine had inhibited the reflex, recovery passed through a stage in which the frequency of the peristaltic waves was increased. As in the experiments on the rabbit's intestine the action of nupercaine was delayed and prolonged: when it was kept in the bath for 3 min. the full effect only occurred a few minutes after it had been washed out.

At the beginning of an experiment tubocurarine, even in very strong concentrations, caused only slight acceleration of the frequency of the peristaltic waves; after several hours twentyfold weaker concentrations might abolish the reflex. The effects like those of cocaine were quickly reversible after washing out the drug. The difficulty of obtaining comparable responses on repeated administration of the same amount of tubocurarine, or graded responses on administration of varying amounts, made the guinea-pig's intestine preparation unsuitable for the quantitative assay of curare-like substances.

In Fig. 12 the changes in sensitivity to tubocurarine chloride are illustrated. The drug was added each time 2 min. before the pressure in the lumen was raised. At the beginning of the experiment 2,000 μg. increased the frequency of the peristaltic waves from 10 to 13 per min. (at *b*); 2½ hours later 600 μg. greatly inhibited the reflex (at *c*), and after a further 3½ hours 100 μg. practically abolished it (at *d*).

The paralysing action of local anaesthetics and of tubocurarine on the peristaltic reflex is much weaker than that of nicotine in paralysing doses,

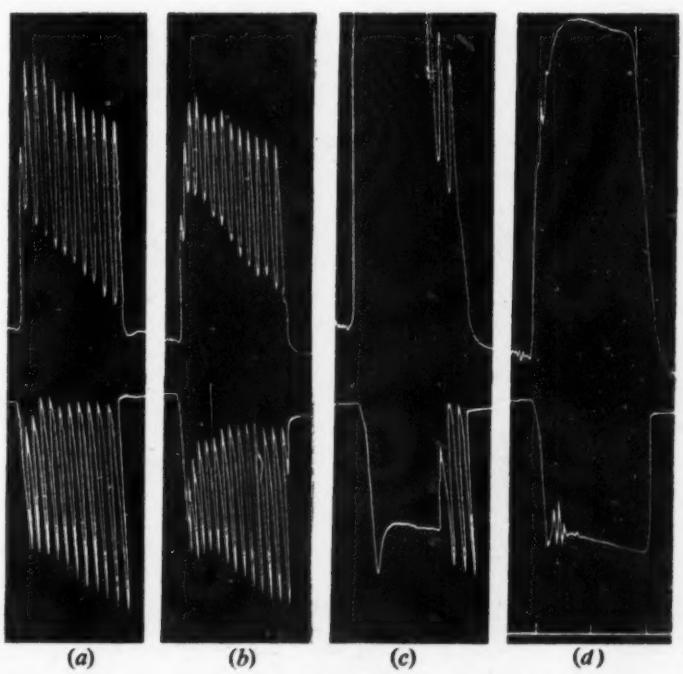


FIG. 12.—Guinea-pig's ileum in 35 c.c. Tyrode's solution. Gradual increase in the inhibition of the peristaltic reflex by *d*-tubocurarine chloride. At (*b*) 2,000 μg., at (*c*) 600 μg. two and a half hours later, at (*d*) 100 μg. six hours later. Time in 30 sec. Details in text.

particularly when the comparison is made on a fresh preparation. An analogous result has been obtained and referred to previously for the inhibition of the stimulating action of nicotine.

In the experiment of Fig. 13, 2,000 μg. of *d*-tubocurarine chloride had no inhibiting action; 600 μg. of nicotine, however, abolished the reflex. The nicotine was added to the bath between *a* and *b* whilst the pressure in the intestinal lumen was low; an immediate strong contraction of the longitudinal muscle occurred, but within 1 min. the muscle relaxed again although the nicotine was kept in the bath. At this stage raising the pressure in the lumen no longer initiated the peristaltic reflex (at *b*). In fact, both the longitudinal and the circular muscle layer were in an atonic condition. Instead of the usual strong contraction of the longitudinal muscle in response to the increased pressure in the lumen there was only a slight delayed shortening of the preparation, and the lack of tone of the circular muscle led to the great filling of the intestine with saline solution. This is seen on the volume record by the profound fall which is followed by a horizontal line because the maximal possible reduction in volume of the Brodie recorder has been reached. Between *b* and *c* the nicotine was washed out; before the reflex returned, the muscle tone was restored in both layers. With the return of the reflex the peristaltic contractions of the circular muscle were at first not sufficiently strong to overcome the contraction of the longitudinal muscle as will be seen from a comparison of *e* with *a* and *f*.

If the nicotine had remained in the bath the return of muscle tone seen at *c* and *d* in Fig. 13 would nevertheless have occurred but the abolition of the peristaltic waves would have remained. In the early stages of nicotine paralysis, but not later, the muscle layers are thus unable to develop tone in response to increased pressure in the lumen and in this condition they have also been found to be less excitable to drugs like acetylcholine or histamine (Cantoni and Eastman, 1946; Emmelin and Feldberg, 1947).

Discussion

On the isolated intestinal preparation it can easily be shown that the response of the intestinal wall to increased pressure in the lumen consists of two phases: a contraction of the longitudinal muscle and a wave of contraction of the circular muscle spreading aborally over the whole preparation. Trendelenburg has aptly termed the two responses the preparatory and the emptying phase of the peristaltic reflex. Only the peristaltic wave of contraction of the circular muscle is nervous in origin and therefore very susceptible to lowering of the bath temperature, to fatigue, and to drugs which affect nerve fibres or nerve cells in the intestinal wall. The contraction of the longitudinal muscle, on the other hand, is a response of the muscle fibres themselves to the stimulus of stretching and is therefore not abolished when the function of the nervous structures in the intestinal wall is interfered with by local anaesthetics or by tubocurarine. In the rabbit's intestine with its pronounced rhythmic contractions of the longitudinal muscle there occurs in addition, even before the pressure in the lumen is sufficiently raised to initiate the shortening of the preparation, an augmentation of the amplitude of the rhythmic contractions. This response also is myogenic in origin. We do not know whether similar myogenic responses to stretching occur in the circular muscle layer. This muscle certainly offers some resistance to the stimulus of filling the gut, and this resistance is not abolished when the nervous structures in the intestinal wall are inactivated by cocaine or tubo-

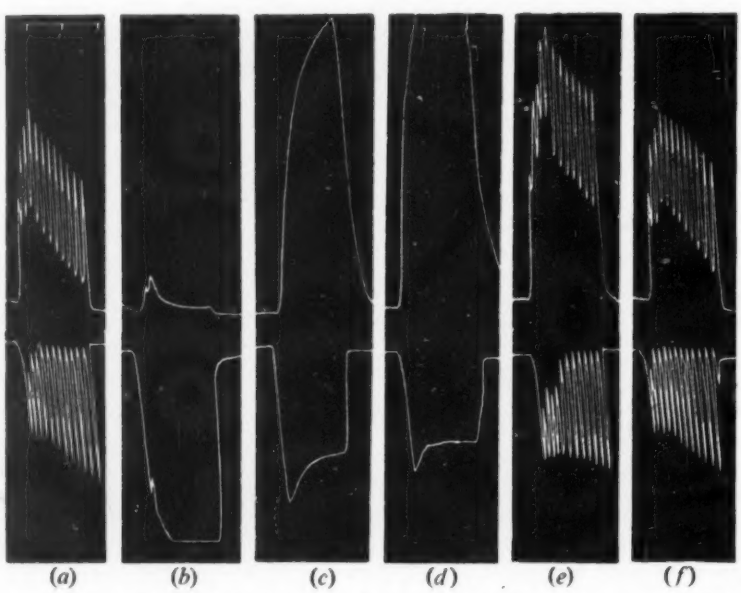


FIG. 13.—Guinea-pig's ileum in 35 c.c. Tyrode's solution. Nicotine paralysis of the peristaltic reflex. Between (*a*) and (*b*) 600 μg. nicotine tartrate were given and washed out at the end of (*b*). Time in 30 sec. Details in text.

curarine, but it is abolished when the muscle fibres are in an atonic condition as, for instance, in the early stages of nicotine paralysis. In addition we have found* that the whole layer of the circular muscle may be thrown into powerful rhythmic contractions at the same frequency as that of the rhythmic contractions of the longitudinal muscle, when eserine in a concentration of about 1 in 300,000 is added to the bath during a period of increased pressure in the lumen and whilst the peristaltic waves of contraction are abolished by cocaine or tubocurarine.

A study of the peristaltic reflex as well as of the simple contractions of the longitudinal muscle on the intestinal preparation reveals the fact that a careful analysis is always necessary before the effects of drugs can be attributed to a nervous or to a muscular site of action. For instance, the shortening of the intestinal preparation when the ganglion cells are stimulated by nicotine does not differ much from the contraction produced by muscle-stimulating drugs, such as histamine or acetylcholine. On the other hand, if the pressure exerted on the intestinal wall from the lumen is just insufficient to initiate the reflex, any drug which increases muscle tone may indirectly elicit the reflex. It is therefore not surprising that drugs which stimulate smooth muscle are known to augment, whereas drugs which relax them are known to inhibit, peristalsis. Straub and his co-workers applied Trendelenburg's technique to the intestine *in situ* and observed increased peristalsis with choline, acetylcholine, and physostigmine, and inhibition of the peristaltic reflex with adrenaline and ephedrine (Straub and Viaud, 1933; Straub and Leo, 1933; Leo, 1933; Straub and Stefánsson, 1937).

If drugs are known to have local anaesthetic or curare-like actions the abolition of the peristaltic reflex in the isolated rabbit's intestine provides a useful reaction for assaying their potency, provided that the concentrations used do not affect the muscle fibres. Nupercaine was found to be about 12 times as active as cocaine in abolishing the peristaltic reflex; this does not necessarily prove a similar relationship for the potency of the two drugs as local anaesthetics, since with different tests widely different results are obtained (see Goodman and Gilman, 1943).

When the peristaltic reflex is used as a reaction for assaying curare-like substances we compare their effectiveness in paralysing autonomic ganglia and not motor endplates. The two effects do not run parallel, as shown by Bovet, Depierre, and de Lestrange (1947) and by Depierre (1947). In previous experiments of this kind the paralysing effects on sympathetic ganglia were compared with those on motor endplates. The ganglia involved in the peristaltic reflex, however, probably belong to the parasympathetic nervous system, and their sensitivity to a curare-like acting substance may be different from that of sympathetic ganglia. The problem appears to be even more complicated since motor endplates of muscles from different species do not show the same sensitivity to different curare-like substances (Wien, 1948).

The greater frequency of the peristaltic waves after cocaine in the freshly suspended guinea-pig's intestine is difficult to explain. When at the same time the waves become incomplete, so that they affect parts of the preparation only, they resemble rhythmic contractions of myogenic origin. But they are not accounted for in this way, for the following reasons: (1) All grades of increased frequency may occur after cocaine. The frequency of the contractions may increase slightly or the rate may double or increase even more. If cocaine were to abolish the peristaltic waves and reveal myogenic contractions there would be only two frequencies—that of the peristaltic waves before, and that of the myogenic contractions after, cocaine. (2) When the contractions do not become incomplete after cocaine, they, like typical peristaltic waves, start at the oral end and spread over the whole preparation.

The frequency of the peristaltic contractions is to some extent dependent on the degree of pressure exerted in the intestinal lumen and increases with the pressure (Trendelenburg, 1917). Cocaine might be thought to produce a similar effect in the guinea-pig's intestine simply by increasing muscle tone. It is more likely, however, that cocaine, and to some extent other local anaesthetics, alter the excitability or the conductivity of either the muscle fibres or the nervous elements or of both structures in the intestinal wall of the guinea-pig in such a way as to speed up the rate of the rhythmic response which occurs when the pressure in the lumen is increased. This action could be grouped then with the well-known central stimulating actions of cocaine. To assume a similar action for tubocurarine would be strange; but its effect in increasing the frequency of the peristaltic waves is only very weak and it may have to be explained in a different way.

SUMMARY

1. Cocaine has stimulating and inhibiting effects on the excitability of the muscle fibres of the

*Unpublished experiments.

rabbit's and guinea-pig's intestine. *d*-Tubocurarine has but slight effects of this kind on these preparations.

2. It is possible to inactivate the nervous structures in the intestinal wall with cocaine or with *d*-tubocurarine. In concentrations which do not yet depress the excitability of the muscle fibres these substances inhibit the responses to small doses of nicotine as well as the peristaltic reflex.

3. The peristaltic reflex initiated by raising the pressure in the lumen of the isolated intestine (Trendelenburg's method) consists of two phases: (i) a contraction of the longitudinal muscle which is a response of the muscle fibres themselves to the stimulus of stretching, and (ii) waves of contractions of the circular muscle layer spreading aborally over the whole preparation and being nervous in origin. Only the latter are abolished when the nervous structures in the intestinal wall are paralysed by local anaesthetics or by tubocurarine. In the rabbit's intestine the familiar strong rhythmic contractions of the longitudinal muscle (pendular movements) also remain unaffected under these conditions.

4. In the guinea-pig's intestine local anaesthetics and *d*-tubocurarine exert their inhibiting effect on the peristaltic reflex only several hours after the preparation has been suspended in the bath. In the freshly suspended preparation the effect of these drugs is to increase the number of peristaltic waves per minute. This effect is strong with cocaine but weak with tubocurarine.

5. Inhibition of the peristaltic reflex in the isolated rabbit's intestine provides a useful reaction for assaying quantitatively the potency of local anaesthetics or curare-like substances, because the inhibition is easily reversible, and comparable effects are obtained on repeated administration of the same dose of these substances, and graded responses with different doses. By this method nupercaine was found to be about 12½ times as active as cocaine.

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THE EFFECT OF N-METHYLATION ON CERTAIN IMINAZOLINES

BY

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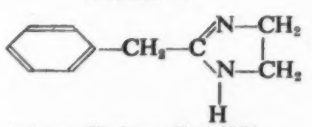
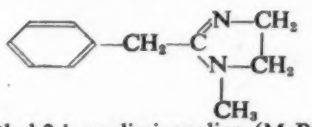
In a previous paper (Gowdey, 1948) it has been shown that methylation of a nitrogen atom in the iminazoline ring of 2-benzyliminazoline (BI) produces important changes in its pharmacological activity. Thus the N-methyl derivative, instead of causing a fall of blood pressure through peripheral vasodilatation, caused a rise of blood pressure, but had no direct effect on vessels, and the pressor effect was shown to be due to a nicotine-like liberation of adrenaline from the adrenals. On the other hand, N-methylbenzyliminazoline had the same qualitative action as BI itself on cardiac tissue, the intestine, skeletal muscle, and the superior cervical ganglion.

Because of the sudden appearance of a nicotine-like vascular activity of the BI molecule when it contained an N-methyl group, it was decided to compare several more 2-substituted iminazolines with their N-methyl derivatives. The structural formulae of the compounds investigated, and the abbreviations which will be used in referring to them, are given in Table I.

TABLE I



2-benzyliminazoline (BI)



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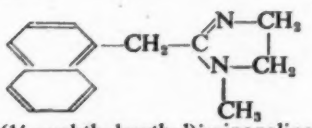

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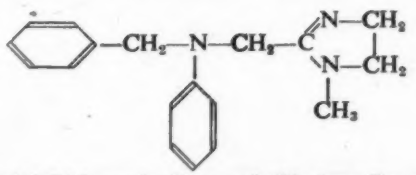
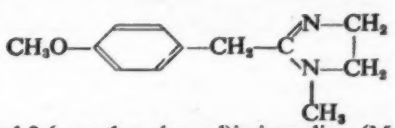


N-methyl-2-(N-benzylanilinomethyl)iminazoline (MeBAI)



N-methyl-2-(*p*-methoxybenzyl)iminazoline (MeMeOBI)

The vascular activity of 2-(1′-naphthylmethyl)-iminazoline (NI) has been studied by several workers (Hartmann and Isler, 1939; Meier and Muller, 1941; Emerson, 1944; Craver *et al.*, 1944; Yonkman *et al.*, 1945; Meier and Bucher, 1946a). 2-(N-Benzylanilinomethyl)iminazoline (BAI), although studied mainly as an antihistamine compound, was tested for other activities by Meier and Bucher (1946b) and by Craver *et al.* (1948). *p*-Methoxybenzyliminazoline (MeOBI) was investigated by Hartmann and Isler in their early paper on the iminazoline series (1939). None of the N-methyl derivatives except MeBI has been mentioned in the literature.

Experimental Results

A. *Action on the cardiovascular system*

(1) *Naphthylmethyliminazoline (NI) and MeNI.*—The intravenous injection of 25–100 μg. NI into a spinal cat causes a large rise of blood pressure, but no change in heart rate. The same dose repeated several times causes less rise of blood pressure than the first dose. The pressor effect of NI is not abolished but it may be reduced by large doses of nicotine or tetraethylammonium iodide or by adrenalectomy. Fig. 1 shows the large rise of blood pressure of the spinal cat produced by the injection of 1 mg. NI at C compared to the rise produced by 20 μg. adrenaline at A

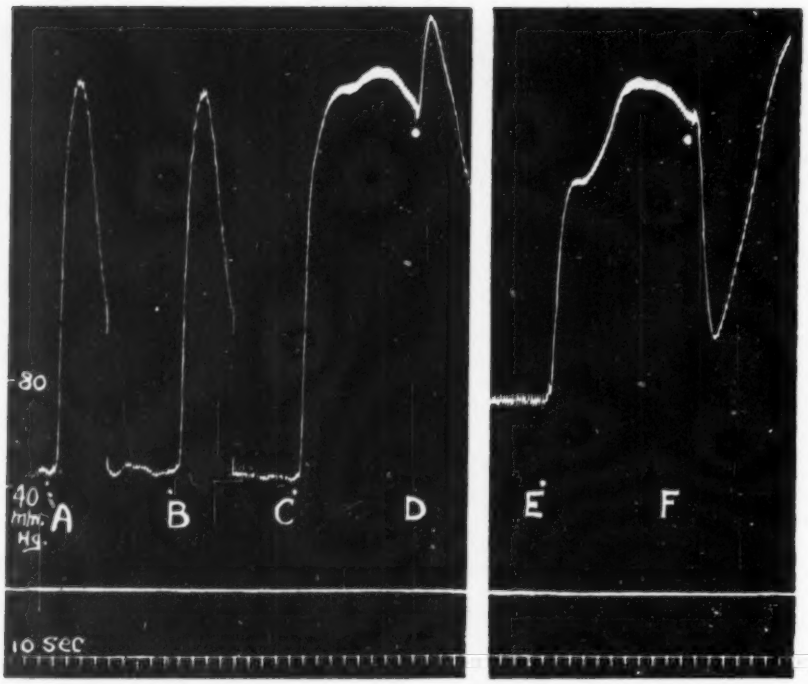


FIG. 1.—Spinal cat. Injections A, B, 20 μg. adrenaline; C, 1 mg. NI; D, 20 μg. adrenaline; E, 20 mg. NI; F, 20 μg. adrenaline.

and B. It also shows that a large dose (20 mg.) of NI (injected at E) reversed the pressor action of 20 μg. adrenaline (injected at F). Thus NI, although a pressor substance, reverses the action of adrenaline in large doses. Both ergotoxine and BI abolished the rise of blood pressure caused by NI, but neither reversed it.

Doses of 25–100 μg. MeNI have no appreciable effect on the blood pressure, but large doses (1–2 mg.) cause a rapid rise accompanied by an increased heart rate. That this pressor action of MeNI was due to a nicotine-like liberation of adrenaline was shown both in the spinal cat and in the cat under chloralose anaesthesia. Large doses of nicotine abolished this pressor response. It was also found that large doses of MeNI (5–10 mg.) injected during the " nicotine paralysis " caused a fall of blood pressure which was later shown to be due to cardiac depression.

Unlike BI and MeBI, which stimulated the isolated perfused cat's heart, NI and MeNI depressed the heart. Fig. 2 (top) shows the effect of injecting 0.25 mg. NI at the 2nd arrow; the heart rate decreased by eight beats/min., the coronary flow from 5.2 to 4.2 ml./min., and the force of contraction by 40 per cent. At the 1st arrow 0.25 mg. MeNI caused a similar decrease in heart rate and coronary flow, but less reduction in amplitude. In the isolated perfused rabbit's heart NI caused great depression, but in the dog heart-lung preparation injections up to 10 mg. had no appreciable effect on cardiac rate, output, or volume.

NI caused vasoconstriction in the rabbit ear perfused by Locke's solution, and in the dog's hindleg perfused with blood containing heparin by means of a Dale-Schuster pump. The powerful vasocontrictor action of NI is absent in its N-methyl derivative. Thus in the perfused rabbit's ear 2 mg. MeNI had no effect, whereas 2 μg. NI caused a long-lasting vasoconstriction. Thus the unsubstituted compound is

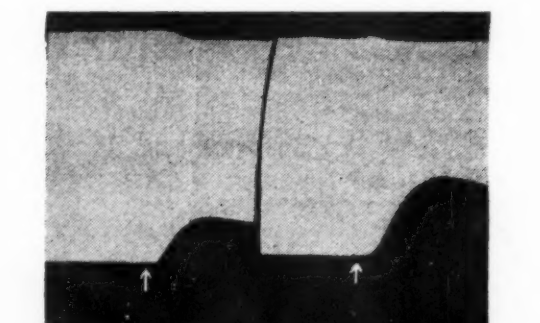


FIG. 2.—Cat heart, Langendorff preparation. Upper record, 1st arrow, 0.25 mg. MeNI; 2nd arrow, 0.25 mg. NI. Lower record, 1st arrow, 0.25 mg. MeBAI; 2nd arrow, 0.25 mg. BAI.

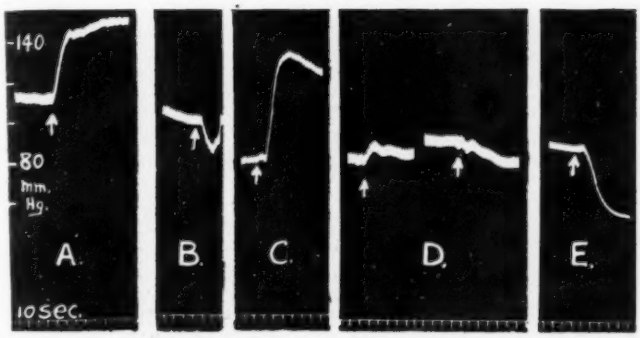


FIG. 3.—Cat, chloralose. A, 5 mg. MeMeOBI; B, 10 mg. MeOBI; C, 2 mg. MeBAI; between C and D, 19 mg. nicotine acid tartrate injected; in D, first 5 mg. MeMeOBI, second 2 mg. MeBAI. E, 2 mg. BAI.

over 1,000 times as active a vasoconstrictor as its N-methyl derivative in this preparation.

(2) *Benzylanilinomethyliminazoline (BAI) and MeBAI.*—Fig. 3C shows the abrupt rise of the cat's blood pressure produced by injecting 2 mg. MeBAI; the same dose of the unsubstituted compound injected at E caused a long-lasting depressor response. After large doses of nicotine the pressor response to 2 mg. MeBAI (injected at 2nd arrow in D) was abolished. Thus it was concluded that the rise of blood pressure caused by MeBAI was also a nicotine-like effect.

Both BAI and MeBAI depressed the isolated perfused cat's heart. The effect of injecting 0.25 mg. of those compounds is shown in Fig. 2 (bottom). The reduction in heart rate and force of contraction was similar to that caused by the naphthyl compounds, but the coronary flow was first increased by the benzylanilino compounds before the longer-lasting reduction occurred.

In the isolated perfused rabbit's ear it was found that the injection of 1 mg. BAI or MeBAI produced definite vasodilatation. The N-methyl compound seemed somewhat weaker in this respect than the parent substance.

(3) p-*Methoxybenzyliminazoline (MeOBI) and MeMeOBI.*—These compounds will be seen (Fig. 3) to have the same general action on the cat's blood pressure as BAI and MeBAI. Thus 5 mg. MeMeOBI injected at A caused a rise of blood pressure and increased the heart rate; this pressor response was no longer significant when the same dose was injected, at the first arrow in D, after nicotine; 10 mg. MeOBI caused a fall of blood pressure when injected at B. The depressor action of MeOBI is not the same as that of BAI, however, because it was found that 10–20 mg. MeOBI injected into a spinal cat reversed the pressor effect of small doses of adrenaline in the same way as BI had done. Further experiments showed that very large doses of adrenaline caused a pressor effect after MeOBI as they had been shown to do after BI.

The isolated perfused cat heart was depressed by both MeOBI and its N-methyl derivative. In this respect the methoxybenzyl compounds were unlike BI and MeBI.

B. *Action on the intestine.*—The normal tone of the isolated guinea-pig ileum was reduced by NI, BAI, and MeBAI, by MeOBI, and MeMeOBI. These compounds also antagonized the contractions induced by acetylcholine. NI in a concentration of 4×10^{-6} was shown to reduce greatly the histamine-induced ileal contraction (see Fig. 4A). On the other hand, MeNI (5×10^{-7}) produced a slowly developing contraction of the ileum which was slightly less than that caused by acetylcholine (2×10^{-5}). If an insufficient interval was left between doses of MeNI, the stimulating

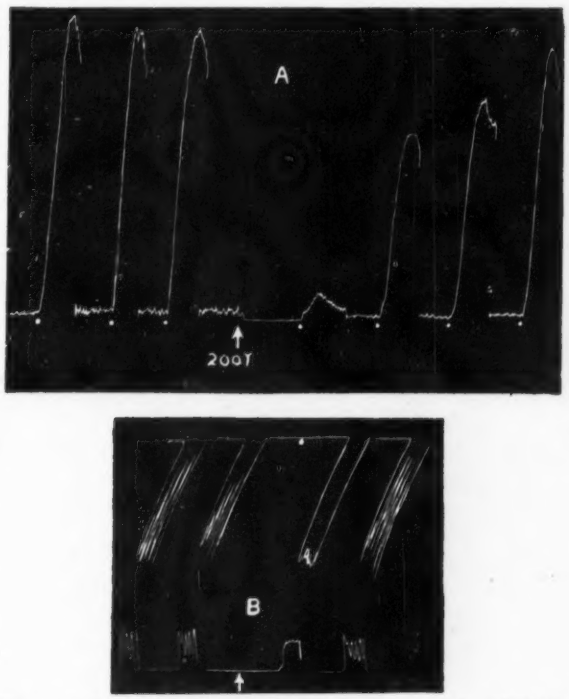


FIG. 4.—Guinea-pig ileum, 50 ml. bath Locke's solution. A: 0.2 μg. histamine at each dot; 0.2 mg. NI at the arrow. B: Trendelenburg preparation, upper record showing peristalsis, lower record longitudinal contractions. At arrow MeMeOBI was added to bath so that the concentration was 4×10^{-6}, addition being made 2 min. before the next stimulus.

effect was reduced or lost completely, presumably because nicotine-like paralysis occurred.

The pendulum movements of the isolated rabbit's duodenum were inhibited by NI in a concentration of 10^{-7}, about ten times the dose of adrenaline required to produce the same effect. MeNI in a concentration of 2×10^{-6} increased the tone and rhythm of the intestine, and prevented the inhibitions regularly caused by 4×10^{-6} NI.

In the Trendelenburg peristalsis preparation as modified by Feldberg and Lin (1948) NI was found to have no effect on the peristalsis, but it inhibited the longitudinal contractions of the ileum. BAI, MeOBI, and its N-methyl derivative inhibited both sets of muscle fibres. The inhibitory effect of 4×10^{-5} MeMeOBI on the circular and longitudinal muscles of the ileum is shown in Fig. 4B.

C. *Action on skeletal muscle.*—All these iminazolines, substituted or not, caused a curariform block in the transmission of the nerve impulse in the phrenic nerve-diaphragm preparation of the rat.

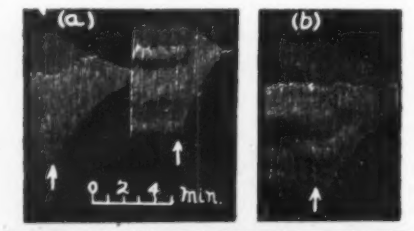


FIG. 5.—Rat phrenic nerve-diaphragm preparation. Stimulation with maximal shocks 6–12/min. Bath 50 ml. T = 36° C. (*a*) 1st arrow, 10 mg. NI; 2nd arrow, 10 mg. MeNI. (*b*) 10 mg. BAI. (*c*) 10 mg. MeMeOBI. (*d*) 10 mg. MeOBI. (*e*) MeBAI. All substances allowed to act for 5 min.

Fig. 5 illustrates this action; all the compounds were given in a final concentration of 2×10^{-4}. Although slight differences in potency were observed, the curarizing effects of these compounds were all of the same order of magnitude.

Curariform activity was also observed in the sciatic-gastrocnemius preparation of the cat. Fig. 6 shows the decreased muscle tension produced by the intra-arterial injection of the various N-methyl derivatives. The parent compounds have the same curarizing action and with doses of the same order of magnitude. That this was a true curariform action and not a direct effect on the nerve or on the muscle was shown by the following experiments. The intra-arterial injection of a large dose

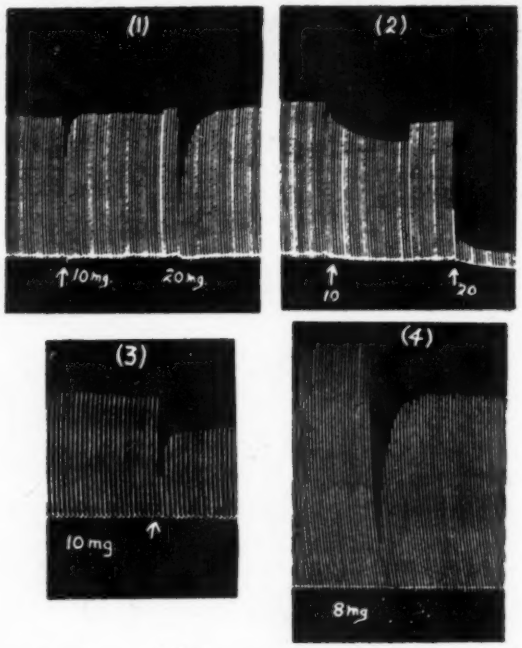


FIG. 6.—Cat, chloralose, left sciatic-gastrocnemius preparation; record of gastrocnemius contraction with tension lever. Injections made into aorta at the bifurcation through the stump of the right external iliac artery. (1) 10 mg. and 20 mg. MeMeOBI; (2) 10 mg. and 20 mg. MeBAI; (3) 10 mg. MeNI; (4) 8 mg. MeBI.

(20 mg.) of MeNI into the fully curarized, directly stimulated gastrocnemius of the cat did not alter the tension developed in the muscle. Nor did MeNI in a concentration of 10 mg./ml. applied directly to the nerve of the isolated frog's nerve-muscle preparation cause any change in the twitch tension evoked by stimulation through the nerve.

D. *Action on the perfused superior cervical ganglion.*—Since the N-methyl compounds of this series shared the nicotine-like pressor action of MeBI, it was expected that they might, like MeBI and nicotine, stimulate the ganglion before depressing its response to preganglionic stimulation. But although depression or abolition of ganglionic transmission occurred with all the compounds, N-methyl-substituted or not, none of them caused a stimulation of the ganglion cells on injection as MeBI had done. A typical example of the curari-

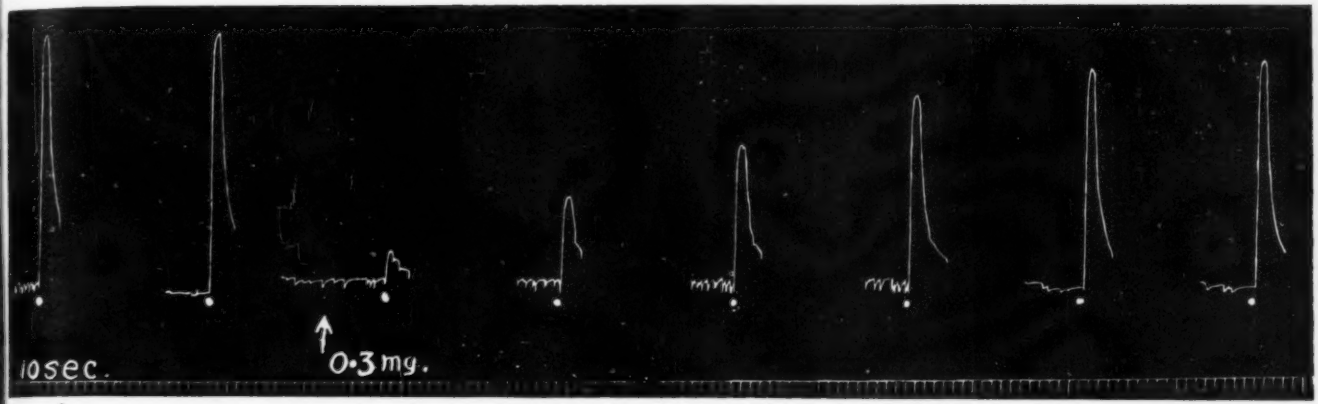


FIG. 7.—Cat, chloralose. Right superior cervical ganglion perfused. Contractions of nictitating membrane in response to supra-maximal stimulation of preganglionic fibres. At arrow 0.3 mg. MeMeOBI injected into the fluid perfusing the ganglion. Note depression of effect of subsequent stimulation.

form block in transmission is shown in Fig. 7; 0.3 mg. MeMeOBI had no effect by itself, but decreased the contraction of the nictitating membrane caused by maximal preganglionic stimulation.

DISCUSSION

The striking thing about this series of iminazolines is that, regardless of the actions of the unsubstituted compounds, their N-methyl derivatives without exception cause a rapid rise of blood pressure and an increased heart rate. The pressor effects have been observed in spinal cats and in pithed cats; therefore the action is not due to stimulation of centres in the cord or brain. From this and the fact that large doses of nicotine abolish the blood-pressure rise, it is concluded that the N-methyliminazolines liberate adrenaline from the adrenals. The pressor action does not appear to depend on a simultaneous stimulation of sympathetic ganglia, for when these substances are injected into the perfused superior cervical ganglion they do not exert a stimulant effect like nicotine (Feldberg and Vartiainen, 1934), BI, and MeBI (Gowdey, 1948). Other substances such as pilocarpine and histamine are already known to liberate adrenaline from the suprarenal glands without stimulating the sympathetic ganglia at the same time. The action of the iminazolines on the ganglion therefore resembles that of tubocurarine rather than that of nicotine, and it is interesting that the curariform activity of the N-methyl compounds is shared by the unsubstituted compounds, as that of nicotine is shared by lobeline, cytisine, etc. Ganglionic transmission is also blocked by the tetraethylammonium ion as well as by tubocurarine and the iminazolines, but the nerve-muscle experiments again suggest that the iminazolines are acting more like tubocurarine than like tetraethylammonium, since the latter augments the contractions of the rat diaphragm (Barlow and Ing, 1948) whereas tubocurarine and the iminazolines alike reduce them.

Of this series only BI and MeBI stimulate the isolated cat heart; the other compounds have a depressant action in the same dose range. In the isolated vessels of the rabbit's ear BI produces vasodilatation; NI produces vasoconstriction, yet their N-methyl derivatives have no effect in a dose 1,000 times as great. Thus N-methylation of the 2-iminazolines causes a shift in their site of action, so that their effect on the blood pressure is now not a direct effect on the peripheral vascular system but is the resultant of the liberation of adrenaline and their direct action on the heart.

Although MeNI stimulated the isolated intestine, the other compounds of this series, whether substituted or not, caused inhibition of the tone, and of the contractions induced by acetylcholine. Experiments with the Trendelenburg preparation showed that the benzylanilinomethyl and *p*-methoxybenzyl compounds also prevented peristalsis. Feldberg and Lin (1948) showed that tubocurarine abolishes peristalsis in this preparation, and since these compounds have a curariform action the inhibition they cause may be due to this property.

SUMMARY

The actions of 2-(1′-naphthylmethyl)iminazoline, 2-(N-benzylanilinomethyl)iminazoline, and *p*-methoxybenzyliminazoline on the blood pressure,

on the isolated heart and perfused vessels, on the intestine, and on ganglionic and neuromuscular transmission have been compared with those of their N-methyl derivatives.

N-methylation was found to cause a shift in the site of action so that the effect of these compounds on the blood pressure was not due to a direct action on the peripheral vascular system but was the resultant of more central actions: stimulation of sympathetic ganglia, liberation of adrenaline, and direct effect on the cardiac muscle.

Nicotine-like activity on the isolated intestine was shown only by N-methyl-(naphthylmethyl)-iminazoline; the other compounds inhibited the tone, and antagonized the contractions evoked by acetylcholine. (N-Benzylanilinomethyl)iminazoline, *p*-methoxybenzyliminazoline, and their N-methyl derivatives had a curariform effect on intestinal peristalsis.

All the compounds of this series, whether N-methylated or not, showed curariform activity on the superior cervical ganglion and on neuromuscular transmission.

I am indebted to Dr. W. F. Short and Dr. P. Oxley, of Boots Pure Drug Co., for the compounds used in this study; I also wish to thank Professor J. H. Burn and Dr. G. S. Dawes for their guidance and encouragement.

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THE TOXICITY OF VESICANTS AND SOME OTHER COMPOUNDS TO THE PYRUVATE OXIDASE SYSTEM* (BRAIN)

BY

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The pyruvate oxidase system in pigeon brain tissue is known to be delicate and sensitive to the tissue inhibitors: fluoride, iodoacetate, and phloridzin (Peters and Sinclair, 1934; Peters, Rydin, and Thompson, 1935). During the war it was used as a test system in Chemical Defence Research during the course of a planned investigation, the origin and trend of which has been described elsewhere (Peters, 1947). The fundamental postulates were that vesication could be initiated by damage to an enzyme with the pyruvate oxidase system as a leading string, and that the attack was upon a sulphydryl grouping; these originated in the earlier finding (Peters, 1936) that dichlorodiethylsulphone (HO_2) (as well as arsenite) had a selective action on the lactate oxidase system in brain, poisoning it at the pyruvate stage. There was a close analogy with the action of iodoacetate, also a vesicant.† At the outbreak of war the researches described here developed simultaneously with the arsenical investigation (Peters, Stocken, and Thompson, 1945) by a team which included E. Holiday, A. G. Ogston, J. St. Philpot, and L. A. Stocken; the objective was a better understanding of the vesicant action and the hope of more effective therapy.

In examining the suitability of the pyruvate oxidase system in brain as a test enzyme its behaviour to several substances allied to mustard gas (H) was carefully investigated; some comparisons were also made with other enzymes. It was found that there was some correspondence between the toxicity of many non-arsenical vesicants to the pyruvate oxidase system and their potency as vesicants, but that this relation failed for H; even so, the enzyme system was still sufficiently sensitive to the latter to be used as a test agent. It is interesting to note in regard to our observations (Peters and Wakelin, 1946) made also at this time upon the -SH nature of some components of the pyruvate system that G. Barron (1936), in the course of observations upon the inhibitors of pyruvate oxidase from gonococcus, noted its sensitivity to oxygen; also more recently Mann and Quastel (1946) and Dickens (1946) consider that it is the enzyme preferentially attacked in high pressure oxygen poisoning. When taken together this work and that upon the biochemical lesion in aneurin deficiency and in arsenical poisoning from this laboratory emphasize the pharmacological importance of this enzyme system.

The present paper embodies the gist of 5 reports written early in the war (Peters and Wakelin, 1940, 1941) and is divided into sections upon the water soluble and water insoluble substances and observations upon other enzymes.

I. Water Soluble Substances

Experimental methods

Brain brei and dispersion and pyruvate dehydrogenase.—These were prepared as described previously (Kinnersley, O'Brien, and Peters, 1935; Banga, Ochoa, and Peters, 1939; Peters and Wakelin, 1946). Brei and

* This paper was planned to be the initial publication of a series, which includes two by the same authors elsewhere (*Biochem J.*, 1946, **40**, 513, and 1947, **41**, 545); its publication has been delayed through unforeseen circumstances. Though some figures are included which are given in one of the other papers, it has been thought desirable to publish the data upon which they are based, because they indicate the kind of accuracy obtainable in this type of pharmacological enzyme investigation.

† Berenblum, Kendall, and Orr (1936) found that mustard gas was more toxic to the whole glycolytic process in tissue than to respiration.

slice experiments were made with oxygen in the bottles, and dispersion experiments with air.

Experiments with the pyruvate oxidase system in pigeon's brain tissue can be made with tissue prepared in three different ways, as slices, as brei (a mashed preparation), and as a dispersion, made by grinding the brain finely in a mortar under ice-cold conditions. The slice resembles the organized tissue best: in this H. M. Carleton has shown that the cell outlines are still intact (unpublished results). In the brei the cell outlines have been mainly destroyed, though large numbers of nuclei are still intact: though the brei approximates more to a preparation of enzymes, some phosphoric esters still fail to penetrate to the active centres present; for instance, vitamin B_1 will penetrate, whereas its pyrophosphoric ester (cocarboxylase) will not (Peters, 1937). In the dispersions we have an enzyme system sufficiently homogeneous to reduce permeability phenomena to a minimum; this has been produced, however, at the expense of stability and a dispersion is only stable for about 30 min. at 38°; it can therefore be used only for rapidly acting substances. It is not necessarily identical with the so-called "homogenates" (Potter and Elvehjem, 1936; Elliott, Scott, and Libet, 1942). As several of the poisons concerned react rather slowly, the most useful information was obtained with the more stable " brei."

Any uncertainties produced by the residual respiration could have been largely eliminated, if desired, by a preliminary washing of the brei with ice-cold Ringer-phosphate solution (Long and Peters, 1939); the delay which would have been caused by this refinement would have made little difference to the result and was not thought worth while. Most experiments were therefore made with unwashed brei.

Respiration was studied in Barcroft Dixon or Warburg type respirometers in Ringer-phosphate *p*H 7.3, 3.0 ml. per bottle ± Na pyruvate ($1.8 \times 10^{-2}M$); the poison was added last after division of the tissue (approx. 100 mg. pigeon brain " brei "). Each figure quoted in Table I and elsewhere represents the average rate of respiration in μl./g./hr. moist tissue (water content approx. 80 per cent) for the time interval given, excluding the initial period of 12 min. required for equilibration. Temp. 38°. Gas: O_2 or air. One pigeon brain (cerebrum + optic lobes) can be distributed conveniently among 12 bottles. In this series 8 bottles (4 duplicates) were devoted to the values for pyruvate alone and those of pyruvate + poison, and the remaining 4 were used to obtain residual values.

A typical experiment is given (Exp. 1939) in Table I; it confirms the previous finding (Peters, 1936) that HO_2 has a marked toxic action upon this system, and that there is no protection even with a relatively high concentration of vitamin B_1 (1 μg. would be a maximum dose for an avitaminous brain). It will be noticed that the effect of HO_2 appears slowly with these concentrations, even with 100 μg./3 ml. not until after 15 min. have elapsed.

The falling rate of respiration is due largely to the decrease in residual respiration: much work (Peters, 1938) has shown that the extra rate of respiration over the period 30–120 min. forms the most reliable estimate in experiments of this type. This period was therefore used to compare the inhibitory effects.

TABLE I

EFFECT OF HO_2 (100 μg. = 675 μM ($10^{-6}M$) UPON THE OXYGEN UPTAKE OF PIGEON BRAIN BREI IN RINGER-PHOSPHATE *p*H 7.3. SUBSTRATE: Na PYRUVATE (0.018M)

Exp. conditions	O_2 uptake in μl./g./hr. during respiration periods (min.)					Average	
	0–15	15–30	30–60	60–90	90–120	0–30	30–120
(*a*) No addition	1550	1276	903	790	490		
(*b*) + 50 μg. HO_2*	1405	1090	750	437	222		
(*c*) + 100 μg. HO_2*	1280	1040	640	246	162		
(*d*) Na pyr.	3315	3060	2710	2305	1845		
(*e*) Na pyr. + 50 μg. HO_2	3285	2875	2080	1325	956		
(*f*) Na pyr. + 100 μg. HO_2	3130	2340	1450	764	361		
(*g*) Same + 10 mg. vit. B_1	3185	2465	1623	917	431		
DIFFERENCES FOR							
Pyr. alone (*d*)–(*a*)	1765	1784	1807	1515	1355	1780	1559
Same + 50 μg. HO_2 (*e*)–(*b*)	1880	1785	1330	888	734	1832	984
Same + 100 μg. HO_2 (*f*)–(*c*)	1850	1300	810	518	199	1675	509

% change in respiration during	0–30 min.	30–120 min.
50 μg. HO_2	—	−37%
100 μg. HO_2	−9%	−67.5%

* Single observations. Remainder are average duplicates. HO_2 = Dichlorodiethylsulphone.

RESULTS

Table II gives the results obtained for a number of water soluble substances. The 50 per cent inhibition values (ET50, expressed in μM) were estimated by inspection of curves drawn through the known points.

In ordinary experiments of this type the standard deviation of a single observation is c. 3.5 per cent, but in the present work substantial further errors undoubtedly arose from difficulties in getting satisfactory solution of some of the substances used, and from uneven actions of the poisons in the early stages before the brei was properly divided.

TABLE II

SUMMARY OF AVERAGE PERCENTAGE INHIBITIONS IN OXYGEN UPTAKE OF PIGEON BRAIN BREI PRODUCED BY SEVERAL WATER SOLUBLE VESICANTS AND OTHER SUBSTANCES. SUBSTRATE: Na PYRUVATE (0.018M)

Respiration period 30–120 min. No. of experiments in brackets

Substance	Conc. $M \times 10^{-4}$	% change	μM for 50% inhibition
Dichlorodiethylsulphone (HO_2) (5)	0.437 0.875 1.75	−22.7 −48.3 −69.4	90
Trichlorotriethylamine (4)	1.91 4.77 9.55	−35.9 −55.1 −63.7	380*
Divinylsulphone (3) ..	0.29 0.86 2.66	−3.2 −52.7 −92.5	75
Dichlorodiethylsulphoxide (3)	1.9 5.72 15.2 57.2 95.2	+6.5 +7 +6.5 −4 −11	Nil at 572
Phenyl-2-chloroethylsulphone (2)	3.3 4.07 8.15 16.3	+1 −25.2 −48 −61	900
Iodoacetic acid (4) ..	2.16 4.32 5.4	−46.8 −69.5 −60.3	250
Iodoacetamide (4) ..	0.54 1.08 1.78	−51.8 −67.7 −85.7	50
Thiodiglycol† and dihydroxydiethylsulphone† (1)	55 and 43	Nil	Nil at 40

* The methyl bis(2-chloroethyl)amine was found by Peters, Thomoson, and Wakelin (1942) to have 50 per cent toxicity at 1.040 μM.

† Non-vesicant.

A few comments are necessary upon the method of dissolving some of the substances and upon their behaviour.

Dichlorodiethylsulphone (HO_2)

Table II gives values for the last four experiments and for a previous one considered to be reliable. Several earlier experiments were excluded after discovery that solution was apt to be incomplete at a concentration of 1 mg./ml. Ringer-phosphate solution owing to the formation of small glass-like globules of the compound which were difficult to see. For these purposes the best way was to add c. 0.5 ml. Ringer-phosphate to about 3.0 mg. HO_2 in a test tube, warm until the crystals went into solution,and then add up to 6.0 ml. to make 0.1 ml.= 50 μg. HO_2. The solution should then be examined for small transparent globules of HO_2 and, if these are suspected, the whole warmed. As hydrolysis is slow in presence of salt (Peters and Walker, 1923) any slight loss is completely offset by the certainty of complete solution. Three separate solutions were used in getting the results of Table II. Upon the same solution, results for different brains agreed closely.

2 : 2′ : 2″-trichlorotriethylamine

Solutions of the pure hydrochloride (kindly supplied by Sir Robert Robinson and Dr. Mason) were used: any necessary small additions of alkali were previously made to the bottles to compensate for changes in *p*H produced by the hydrochloride. The free base is liberated as an oil at *p*H 7.3.

Divinylsulphone (DVS)

The experiments quoted were done with a pure specimen from Dr. McCombie, supplied through Dr. Fell. A few mg. stirred with appropriate volumes of water (c. 5.0 ml.) appeared to give satisfactory solution.

Phenyl-2-chloroethylsulphone

This compound (supplied by Porton) was dissolved as far as possible by warming. It does not hydrolyse to any extent.

Iodoacetic acid and iodoacetamide

It was noted that the poisoning induced by iodoacetamide in the first 30 min. is relatively greater than that with other compounds, showing that the condensation is very rapid.

Thiodiglycol and dihydroxydiethylsulphone

In this experiment the values given for the O_2 uptake are those of the bottles containing pyruvate and pyruvate + substances added, without subtraction of the residuals. These substances had a depressant effect on the residual respiration whereas they will be seen to have none on the values in presence of pyruvate. This is interpreted to mean that there is competition for the active centres in the brei between the last traces of pyruvate present in the residue and these substances.

In order to get a better picture of the accuracy of these comparisons, the behaviour of HO_2, divinylsulphone, and trichlorotriethylamine was examined more closely. Dr. R. B. Fisher, of this Department, kindly tested the figures for HO_2 and trichlorotriethylamine (Table II) statistically upon the assumption that the relation between percentage inhibitions and log. concentrations is linear. This was more true for HO_2 than for trichlorotriethylamine, owing to the hydrolysis of the latter during the 2 hr. of experiment. The analysis showed a rather wide variance for the figures as they stood; the probable value for the ratio between means at the point of 50 per cent toxicity was 4.3, with a 1/20 chance of the ratio lying outside the limits 2.6 to 7.3.

In view of this, which was due mainly to the difficulty of standardizing the initial rate of action of the poison in the brei, we carried out a few further experiments, making a direct comparison at the molarities required to give approximately 50 per cent inhibition in order to get more information upon the accuracy of this type of experiment. Experiments were made with 16 bottles as follows: two 0 (residual), two + pyruvate, three + HO_2, three + trichlorotriethylamine, three pyruvate + HO_2, and three pyruvate + trichlorotriethylamine. The results are given in Table III.

The statistical estimate made the probable value for the ratio 4.43, with limits of 2.72–7.21 (1/20 chance) in agreement with the estimate in Table II. This gives the possible limits of accuracy of this type of experiment.

Table III (*b*) confirmed the previous figures. On a molar basis divinylsulphone was the most toxic substance of the series.

Since the first object of this work was a comparison of toxicity to the pyruvate system with vesication, the values for enzyme inhibition (50 per cent toxicity) were compared with the Porton data for vesication (upon a weight basis). In Table IV HO_2 is put as 100 and some correspondence between vesicant action and toxicity will be seen. The only marked discrepancy was in the values for divinylsulphone; this is known to be highly toxic to animals by injection, and on the arm of one of us (R.A.P.) small amounts readily formed vesicles. We are inclined to consider the available Porton estimate too low, though could not press this in the absence of further data. So far as the hypothesis of an attack upon the -SH group in the enzyme is concerned, the data given so far are in agreement; HO_2 and divinylsulphone have a μM toxicity (ET50) of 90 and 75 respectively, which is to be compared with the trivalent arsenical toxicity (Peters, Sinclair, and Thompson, 1946) of 17–30 μM, where two -SH groups are attacked per mol. The case is different for mustard gas now to be considered.

TABLE III

COMPARISON OF TOXIC EFFECTS UPON THE PYRUVATE OXIDASE SYSTEM OF THE SAME PIGEON BRAIN BREI TISSUE RESPECTIVELY OF (*a*) HO_2 AND TRICHLOROTRIETHYLAMINE AND (*b*) HO_2 AND DIVINYLSULPHONE (DVS). SUBSTRATE: Na PYRUVATE, 0.018*M*. RESIDUALS SUBTRACTED

(*a*)	Conc. $M \times 10^4$		Average resp. rate in μl./g./hr.		% inhibition	
Exp.	HO_2	Trichloro-triethyl-amine	0–30 min.	30–120 min.	0–30 min.	30–120 min.
1970	—	—	1980	1849	—	—
	0.9	—	1831	901	−7.5	−54.8
1971	—	—	1636	1378	—	—
	0.9	—	1728	766	—	−44.4
	—	3.87	1311	727	−19.5	−47.2
1972	—	—	1636	1542	—	—
	0.9	—	1339	645	−18	−58.2
	—	3.87	1214	712	−26	−53.9

(*b*) HO_2 AND DVS. DIRECT COMPARISON. NO CONTROLS WITH PYRUVATE ALONE. TRIPLICATE ESTIMATIONS. HO_2, $0.91 \times 10^{-4}M$; DVS, $0.74 \times 10^{-4}M$; 52 μg. AND 26 μg. PER 3 ML. RESPECTIVELY

Pyruvate	HO_2	DVS			Differences	
−	+	−	1263	521		
−	−	+	1296	539		
+	+	−	2204	902	941	381
+	−	+	2115	985	819	446

TABLE IV

COMPARISON OF "OXIDASE" TOXICITY AND VESICANT ACTION; $HO_2 = 100$

Substance	Oxidase toxicity	Vesicant action
Iodoacetamide	186	Unknown*
Divinylsulphone (DVS)	198	2.5
Dichlorodiethylsulphone (HO_2)	100	100
Iodoacetic acid	37	Vesicant
Trichlorotriethylamine (hydrochloride)	19.8	10–20
Phenyl-2-chloroethylsulphone	9	nil
Dichlorodiethylsulphoxide	nil	nil
Thiodiglycol and dihydroxydiethylsulphone	nil	nil

* One of us (R.W.) produced on the left forearm a large erythematous patch 8 × 8 cm. and vesicle 3.5 × 6.0 cm. with an application of 1.5 mg. iodoacetamide in ethanol.

II. Water Insoluble Substances

Mustard gas (H)

Some early experiments were done by the method used by Berenblum *et al.* (1936), in which the substance was mixed with the tumour tissue thoroughly before it was added to the respiration bottles. Table V gives some results so obtained.

TABLE V

CONCENTRATIONS* FOR IMMISCIBLE SUBSTANCES AND THIODIGLYCOL GIVING APPROX. 50 PER CENT DECREASE IN O_2 UPTAKE OF PYRUVATE OXIDASE SYSTEM IN PIGEON BRAIN BREI

Substrate: Na pyruvate, 0.018*M*. No. of experiments in parentheses

Substance	Conc. for −50%(μ*M*)	Conc. for −10%(μ*M*)
2:2′-Di(β-chloroethylthio)-diethyl ether	330 (3)	—
Mustard gas	770 (3)	—
Phenyl-2-chloroethylsulphide	1000 (3)	—
Thiodiglycol	—	150 (2)
Butyl chloride (tertiary)	—	200 (3)
Butanol (tertiary)	—	240 (1)

* Concentrations in this series of experiments only are reckoned in terms of the amount of test substance per 100 mg. tissue used.

It will be seen that although H is toxic it is much less so than the arsenicals and divinylsulphone. Thinking that this might be due to the inefficient method of introduction of the poison, we tried other solvents. Table VI gives the results of the early and some later experiments using *iso*propanol and ethyl and methyl cellosolve.

It was possible to use these solvents with a correction, and in fact this was done later in the experiments in this laboratory upon BAL (Stocken, Thompson, and Whittaker, 1947); but it was then thought advisable to find some other method, and the use of lecithin was explored. Commercial lecithin (B.D.H.) cannot be used for the emulsification as it is toxic in itself. Egg lecithin was therefore prepared as follows (we are indebted to Professor J. B. Leathes for the details of this preparation):

Preparation and use of lecithin.—The yolks of 6 eggs were stirred with 3 volumes of acetone and the solution filtered; the solid residue was stirred with a further 3 volumes of acetone. The residue was then shaken with

TABLE VI

TOXIC EFFECT OF SMALL CONCENTRATIONS OF SOLVENTS UPON PYRUVATE OXIDASE SYSTEM AND THE COMBINED EFFECT OF SOLVENT AND MUSTARD GAS. BREI AND DISPERSIONS, PIGEON BRAIN. SUBSTRATE: Na PYRUVATE, 0.018*M* (BREI); 0.011*M* (DISPERSION)

Me.cs. = Methyl cellosolve, 0.025 ml./3 ml. Et.cs. = Ethyl cellosolve. H = Mustard gas. L = Lecithin

Exp.	Substrate	Qo_2 0–30 min.	Averate rate 30–120 min.	Decrease	Dispersion (D) or brei (B)
2212	Pyr. alone	13.05	9.98		B
	Pyr. + *iso*propanol 0.84%	10.78	8.61	1.37	
	Same + H	9.90	6.21	3.77	
2223	Pyr. alone	19.46	11.58		B
	Pyr. + *iso*propanol	14.08	9.22	2.36	
2224	Pyr. alone	16.36	11.43		B
	Pyr. + *iso*propanol	14.30	9.56	1.87	
2226	Pyr. alone	16.90	11.57		B
	Pyr. + propanol	13.50	9.00	2.57	
2227	Pyr. alone	17.01	11.34		B
	Pyr. + *iso*propanol	12.96	9.51	1.83	
	Pyr. + *iso*propanol + 800 μg. H	11.37	5.88	5.46	
2323	Pyr. alone	15.53			D (dialysed)
	Pyr. + Me.cs.	6.21		9.32	
2327	Pyr. alone	9.59			D (dialysed)
	Pyr. + Et.cs.	7.52		2.07	
	Pyr. + Et.cs. + 800 μg. H	5.48		4.11	(extra for H −2.03)
	Pyr. + L	10.30			
	Pyr. + L + H	8.13		2.17	

acetone for 2 hr. in a mechanical shaker, the suspension filtered, and the residue suspended in absolute ethanol overnight. After filtration the residue was again suspended in absolute ethanol and shaken mechanically for 2 hr. The ethanolic filtrates were combined and concentrated *in vacuo* at 38–40° to a small volume and made up so that 1 ml. = 100 mg. When this solution is kept in the ice chest, a deposit of sphingomyelin gradually forms. We have found the product from this relatively crude ethanolic solution quite suitable for our purpose, and it gives no inhibition of respiration with a brei.

For experiment, weighed amounts of H (c. 8 mg.) were added to 50 mg. lecithin (after removal of ethanol *in vacuo* in a small test tube) and an intimate mixture was made with a glass rod. Immediately before being added to the respiration bottles, the emulsion was treated with 0.5 ml. Ringer-phosphate solution with vigorous stirring, followed by a further 1.5 ml. to a volume of 2.0 ml. From this a usual addition to the bottles was 0.2 ml. (5 mg. lecithin).

Experiment 2254 (Table VII) shows that the amount of lecithin added to the 3.0 ml. fluid in the respirometer bottles can be varied from 1–5 mg. without change, but that 10 mg. may reduce the effect of the H. In this experiment the H (50 μg.) was potentiated by addition of diethanol dithiocarbamate (Peters and Wakelin, 1947) (1 mg. per bottle).

TABLE VII

EXP. 2254. INHIBITORY EFFECT OF POTENTIATED H (420 μM) UPON Qo_2 OF PYRUVATE OXIDASE SYSTEM OF PIGEON. BRAIN BREI WITH VARYING AMOUNTS OF LECITHIN PRESENT. Na DIETHANOL DITHIOCARBAMATE (1.9 mM) ALSO PRESENT

Substrate: Na pyruvate, 0.018M

Lecithin	Qo_2	%
+ 1 mg. ..	−4.35	−59
+ 5 mg. ..	−4.62	−62
+ 10 mg. ..	−3.67	−46

We have employed this method of adding oily substances extensively and have confidence in the results obtained. The arguments in favour of the use of lecithin may be summarized:

(*a*) Lecithin is a normal constituent of brain and other tissue; its addition therefore only increases slightly the amount already present in the tissue.

(*b*) When properly purified and added alone, it has no effect upon the Qo_2.

(*c*) It has no competitive action with H (Holiday, Ogston, Philpot, and Stocken, 1940; Ogston *et al.*, 1948) but merely reduces the rate of hydrolysis, as has been proved experimentally by Ogston (personal communication).

(*d*) There is no increased toxicity if the combined emulsion of lecithin and H is allowed to stand before addition of the Ringer-phosphate, or even if it is warmed to 38°; hence it does not form a toxic addition compound. Experiment 2304 shows this (Table VIII).

TABLE VIII

EXP. 2304. BRAIN BREI (PIGEON). Qo_2 FOR PERIOD 35–120 MIN. SUBSTRATE: Na PYRUVATE (0.018M); LECITHIN IN ALL BOTTLES

Additions	Qo_2
No addition	10.45
800 μg. H*	5.89
800 μg. H stood for 1 hr. at room temperature after thorough mixing*	6.18
800 μg. H warmed in bath for 20 min. after thorough mixing*	5.97

* The Ringer-phosphate was added to the thoroughly stirred lecithin immediately before addition to the bottles.

Tables IX, X, and XI give a selection of results obtained for mustard gas with dispersion, with brei, and with slices; these are given separately because there are differences in the detail of the effect. The toxicity (ET50) works out at more than 1,000 μM. This is clearly a relatively low toxicity compared with those of divinylsulphone and the arsenicals, even if we allow for a slight loss by preliminary hydrolysis before the poison has time to act; it is of the same order of toxicity as that of iodoacetic acid to this enzyme.

Dispersion.—The action upon pyruvate respiration (Table IX) is noticeable in the first 10 min. period of observation and fully established after 20 min.; the maximum effect was seen in the respiration period 10–20 min. Only 3 out of 15 experiments showed significant inhibition in the residual respiration. The protocol of 2 typical experiments out of the large number performed are given.

TABLE IX

DISPERSION (PIGEON BRAIN). CHANGE IN RATE OF RESPIRATION IN μl./g./hr. O_2 UPTAKE, DUE TO ADDITION OF LECITHIN + H (800 μg. H IN 5 MG. LECITHIN PER BOTTLE). SUBSTRATE PYRUVATE. RESPIRATION PERIOD IN MIN.

Exp. No.	0–10	10–20	20–30	30–40	Average	% change
2047	−660	−670	−580	−485	−599	−18
2061	−225	−795	−835	−353	−560	−16

The values given represent the decreased rate of O_2 uptake over the periods mentioned, i.e., that for pyruvate minus pyruvate + H; for the reason why this procedure is used see Peters (1938).

Brei.—Fig. 1 shows the rate of poisoning of brei in a typical experiment; Table X gives the percentage inhibitions observed in a series of experiments with different concentrations of H. The lowest amount producing a noticeable change is 100 μg. H/3 ml.; the effect depended much upon the efficiency of emulsification and the rapidity with which the emulsion was added to the bottles. The effect was noticeable in the first 15 min. but did not become maximal until the 30–60 min period. Experiment 1993 shows that, if first allowed to hydrolyse in an aqueous solution, the H produced no action, as is readily understood from consideration of the kinetics (Ogston *et al.*, 1948).

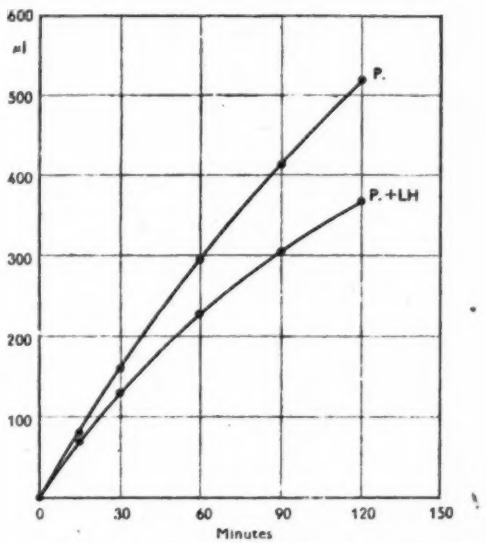

FIG. 1.—The effect of H in lecithin (LH) upon the respiration of brain brei with sodium pyruvate (P) as substrate. Ordinate ml./g./hr. Abscissae min. Pigeon brain brei.

In the final experiments, the changes produced by addition of 800 μg. of H to each bottle (1.67 m*M*) were of the order of −16 per cent for period 0–30 min. and −30 per cent for 30–12 min.—i.e., the concentration required to produce a 50 per cent change was greater than 1,000 μM.

Slices.—Exp. 2065 (Table XI) confirms that in slices too lecithin has no influence on the respiration; H here inhibited the residual respiration. In Exp. 2068 it will be seen that with glucose the poisoning effect is increasing up to 3 hr. Exp. 2072 was a comparison between the action of H with pyruvate and glucose as substrates, in which it will be noticed again that poisoning of the respiration in presence of glucose is increasing up to 3 hr.

TABLE X

BREI (PIGEON BRAIN). CHANGES IN RATE OF RESPIRATION GIVEN IN PER CENT, DUE TO ADDITION OF H IN LECITHIN IN VARIOUS CONCENTRATIONS. SUBSTRATE: Na PYRUVATE, 0.018*M* (residuals subtracted)

Exp.	Amount H per bottle (μg.)	% change	
		0–30 min.	30–120 min.
1987	750	−4.5	−50.1
1988	650	+5	−19
	325	+11	0
1990	750	−22.3	−29.8
1991	750	−16	−43.5
1992	515	−12.6	−23.5
1994	1180	−20	−41.7
	590	+1	−23.5
1995	400	−34.2	−45.2
1997	660	−10.7	−27.5
	330	+3	−9.4
1998	510	−6	−30.7
1999	900	−26.5	−47.8
2000	760	−24.3	−47
2003*	360	−7.5	−23.4
	120	−44	−6.5
2004*	50	−12.3	−17.5
	30	−2	—
2006*	50	−7.8	−12

* Residuals not subtracted

H-lecithin added after hydrolysis in Ringer-phosphate for 30 min. at 38°

1993	1080	−8.2	0

NOTE: In most recent experiments, as the result of further emulsification and quicker working, the changes produced by 800 μg. H/bottle ($1.67 \times 10^{-3} M$) were of the order of −16 per cent for 0–30 min., and −30 per cent for 30–120 min.

DISCUSSION

In a comparison of the rates of poisoning and of hydrolysis for H it is to be noted that in accord with the theory of the kinetics of replacement of chlorine in H (Ogston *et al.*, 1948) any substitution with a group in the enzyme must take place during the hydrolysis; most of this occurs in these experiments in the first 15 min. and is complete in 30 min. The rate of action upon the dispersion was consistent with this; that upon brei and slices was too slow. One conceivable explanation for this difference was conversion of H to an oxidation product; in a direct test of this we could find no increased O_2 uptake owing to the addition of H which might suggest oxidation to sulphoxide. It seems more likely that there is adsorption into inactive lipoid parts of the brei or slice with slower penetration to the active centres; this could explain most of the effect in slices when pyruvate

TABLE XI

SLICES (PIGEON CEREBRAL HEMISPHERES). CHANGES IN RATE OF RESPIRATION DUE TO ACTION OF H IN LECITHIN (L) (800 μg./ml.)

L = Lecithin. Gl. = Glucose. H = Mustard gas. Pyr. = Pyruvate

Exp.	Substrates	Respiration rate in μl./g./hr. during periods (min.)						
		0–15	15–30	30–60	60–90	90–120	120–150	150–180
2065	None (*a*)	1851	1585	1290	976	809	698	
	L (*b*)	1846	1555	1240	967	761	604	
	L + H (*c*)	1670	1406	1055	689	471	307	
	(*b*)–(*c*)	−176	−149	−185	−278	−290	−297	
2068	Gl. only (*d*) ..	—	3350	3255	3255	3247	3147	3057
	Gl. + L (*e*)	—		3320	3135	3275	2990	3000
	Gl. + L + H (*f*) ..	—		2875	2630	2465	2155	1995
	(*e*)–(*f*)			−445	−505	−810	−835	−1005
2072	Pyr. + L (*g*)		4260	3794	3006	2910	2630	2320
	Pyr. + L + H (*h*) ..	3625	3485	2997	2362	2055	1745	1341
	(*g*)–(*h*)		−775	−797	−646	−855	−885	−979
	Gl. + L (*j*)	3720	3785	3650	3445	3500	3340	3265
	Gl. + L + H (*k*) ..	3085	3070	2514	2440	2400	2120	1915
	(*j*)–(*k*)	−640	−715	−836	−1005	−1100	−1220	−1350

is the substrate; it would leave unexplained the gradual increase in poisoning observed with slices in glucose solutions. Since this work was reported in 1940, Dixon and Needham and colleagues (for review see 1946) have produced much evidence in support of the poisoning of hexokinase by H, which would explain the latter effects better. Other factors contributing to the slower action may be the adsorption and half product formation (Peters and Wakelin, 1947) and also perhaps sulphonium salt formation (Stahmann *et al.*, 1946).

III. Selective Action of H

In 1936 (Peters) it was shown that HO_2 poisoned the lactate oxidase system of brei selectively at the pyruvate stage; since Keilin's cytochrome system is common to the oxidation of both lactic acid and pyruvic acid, this experiment indicated that the cytochrome system and the lactate dehydrogenase were much less sensitive to HO_2 than pyruvate dehydrogenase; it also excluded poisoning of cozymase. Though the sulphone combined with glutathione, the latter did not reactivate the poisoned system; except for the more powerful action of HO_2, the effect resembled that of iodoacetate (Peters, Rydin, and Thompson, 1935).

After showing that H could poison the pyruvate oxidase system we thought it advisable to confirm the earlier conclusions for HO_2 by direct experiment and to extend them to H. Direct experiments were therefore done (1) upon the vitamin B_1 component, (2) upon the cytochrome system, (3) upon the total succinate oxidase system in brain, and (4) upon amino-acid oxidase, especially because this contained the adenine flavine dinucleotide as a component.

Aerobic experiments

Cocarboxylase.—It had been shown previously that addition of vitamin B_1 did not stop the poisoning effect of HO_2. In Exp. 1875 (Table XII) it was shown that addition of large amounts of cocarboxylase, now known to be the active component (Banga, Ochoa, and Peters, 1939), does not restore the poisoned respiration.

Cytochrome system.—Cytochrome oxidase and cytochrome C were prepared by the method of

TABLE XII

SEPT. 6, 1939. FAILURE OF THE ADDITION OF COCARBOXYLASE TO RESTORE ACTIVITY OF PYRUVATE OXIDASE SYSTEM POISONED BY DICHLORODIETHYLSULPHONE (HO_2). PIGEON BRAIN DISPERSION; SUBSTRATE: PYRUVATE, 0.018*M*

HO_2 μ*M*	Cocarboxylase μg.	O_2 uptake μl./g./hr. (20 min.)	% change
—	—	2645	
100	—	2242	−15%
100	18	2242	−15%

0.2 μg. cocarboxylase should produce a maximum effect under these conditions in a vitamin B_1 deficient brain.

Keilin and Hartree (1938), and hydroquinone was used as substrate. Table XIII gives the results.

Arsenite, HO_2, and divinylsulphone, all of them vesicant or potentially vesicant substances, produced no effect; 10 μg. As_2O_3 poisoned the pyruvate system to the extent of 85 per cent and 25 μg. divinylsulphone, 50 per cent. H (emulsified in lecithin) showed inhibitions up to 7 per cent, when cytochrome C was present in maximum concentration, and up to 20 per cent when the latter was present in submaximal amounts. That this effect was mainly on the cytochrome C component was suggested by two other experiments: in one (Exp. 6) the action upon the cytochrome oxidase, to which cytochrome C was added before mustard gas-lecithin, was compared with the effect when cytochrome C was added after incubation for 15 min. with H; by this time much of the H would have been hydrolysed or rendered ineffective by combination with phosphate, etc.; in another (Exp. 7) cytochrome oxidase was incubated for 15 min. with and without lecithin, the residue centrifuged and tested with cytochrome C. There was no essential difference from the experiment carried out in the presence of the poison; where much cytochrome C was present, the effect was reduced.

TABLE XIII

EFFECT OF MUSTARD GAS, SOME SULPHONES, AND ARSENITE UPON KEILIN'S "CYTOCHROME SYSTEM"

All bottles contained cytochrome oxidase (0.3 ml.); cytochrome C (0.7 per cent solution as stated); 5 mg. hydroquinone in 0.2 ml. ($1.5 \times 10^{-2}M$) was added after equilibration. L = Lecithin. H = Mustard gas. HO_2= Dichlorodiethylsulphone. DVS = Divinylsulphone

Exp.	Cyt. (ml.)	Additions	Time in min.	μl.O_2	% change
1	0.02	None	20	224	
24.4.40	0.02	+ L (7.5 mg.) ..	20	243	
	0.02	+ L + H (1,200 μg.)	20	250	−17.8
	0.02	+ HO_2 (200 μg.) ..	20	248*	
2	0.2	None	30	262	
25.4.40	0.2	+ As_2O_3 (200 μg.) ..	30	265	+1.5
3	0	None	20	344	
7.8.40	0	+ L	20	336	
	0	+ L + H (1,200 μg.)	20	286	−15
	0	+ L + DVS (150 μg.)	20	334	—
4	0	+ L (5 mg.) ..	20	325	
7.8.40	0	+ L + H (800 μg.)	20	292	−11
	0.02	+ L (5 mg.) ..	20	353	
	0.02	+ L + H (800 μg.)	20	328	−7
5	0	+ L	15	249	
8.8.40	0	+ L + H (800 μg.)	15	207	−20†
	0.1	+ L	15	351	
	0.1	+ L + H (800 μg.)	15	343	−2‡
6	0.1	+ L	15	405	
8.8.40	0.1	+ L + H (800 μg.)	15	386	−5
	0.1	+ L	15	420	
	0.1	+ L + H (800 μg.)	15	383	−7§

* Single observation. † Addition of extra cytochrome C reduced the toxicity. ‡ Cytochrome C added before L + H. § Cytochrome C added after 1 min. incubation with L + H.

NOTE: Vessels containing H had 0.05 ml. N/10 NaOH. per 800 μg. to neutralize the acid formed from H.

EXP. 7. Equal amounts of cytochrome oxidase suspension were incubated (A) with lecithin only and (B) with H in lecithin for 20 min.; N/10 NaOH was added at 10 min. to B to adjust the pH. After being centrifuged, aliquot amounts were compared for activity with and without cytochrome C (0.1 ml.).

	Cyto. C	μl.O_2	% change
A	0	292	
B	0	259	−13
A	+	363	
B	+	341	−6.5

These inhibitions of cytochrome C indicate that H can combine with it; they are of no significance in the experiments upon brain brei as the cytochrome C is not a limiting factor; this was tested directly in one experiment 8 where there was no significant change.

EXP. 8. Brain dispersion from the cerebrums of two pigeons was treated with lecithin (L) and H (800 μg.) in lecithin as usual; after 10 min. preliminary incubation period and a further period of 5 min., 0.15 ml. cytochrome oxidase (cyto. ox.) + 0.05 ml. cytochrome C (cyto. C) were added from a dangling tube to see whether this improved respiration. Substrate: Na pyruvate, 0.023 M. Average of duplicates.

Addition	μl.O_2/g./hr. 30 min.	Change
L only	2933	
HL	2622	−311
L+cyto. ox.+cyto. C ..	2780	
HL+cyto. ox.+cyto. C	2452	−328

Succinate oxidase system.—Table XIV is a summary of tests made upon the relative toxicity of these compounds to succinate and pyruvate respiration in the same brei. In each test there is 2–3 times larger inhibition of the pyruvate than that of the succinate respiration; the effect is therefore selective. There is a sporadic slight poisoning of the succinoxidase system, not yet explained, which is connected with the state of the tissue, because in ground muscle preparations the succinodehydrogenase is not poisoned by H.

Exp. 2027 is a striking variation of the same theme. We have shown separately (Peters and

TABLE XIV

COMPARISON OF TOXICITY TO PYRUVATE AND SUCCINATE SYSTEMS. SUMMARY OF CHANGES (INHIBITIONS) DUE TO POISON IN μl./g./hr. FOR PERIOD (2) 30–120 MIN.

Poison*	Exp.	Residual	Pyruvate	Succinate
HO_2 (0.173 m*M*) ..	1893	−221	−1120	−282
H (as oil) (0.592 m*M*)	1932	−167	−696	−273
H (in lecithin) (1.31–1.72 m*M*)	2032	−92	−631	−264
,, ,,	2099	−251	−698	−139
,, ,,	2034	−120	—	−125
,, ,,	2036	−100	—	−341
H (0.315 m*M*) in lecithin and diethyldithiocarbamate (1.56 m*M*)	2027	−346	−1136	0

* Per 100 mg. tissue

Wakelin, 1943, 1947) that diethanoldithiocarbamate potentiates the toxicity of H. Here an amount of H, which would have had no action upon pyruvate oxidation by itself, had a large effect in presence of diethanoldithiocarbamate ; under the same conditions succinate oxidation was unaffected. Since the cytochrome system is common to both oxidations, we have a remarkably clear selective inhibition.

Amino-acid oxidase.—This was prepared from pig's kidney by the method of Krebs (1935). The system employed for study consisted of 2.0 ml. enzyme extract and 1 ml. buffer (*p*H 7.3) with or without additions made up in buffer solution. The enzyme extract was made by shaking 1 g. dry powder with 40 ml. water at room temperature for 10 min. and then centrifuging. Table XV gives the results and shows that none of the poisons tested interfered with the amino-acid oxidase

TABLE XV

EFFECT OF SOME POISONS UPON THE AMINO-ACID OXIDASE FROM KIDNEY

Substances	O_2 uptake (μl.) in 55 min.
No addition	25
dl-Alanine (0.22*M*)	382
,, ,, + 5 mg. lecithin..	394
,, ,, + 700 μg. H in lecithin	394
,, ,, + 200 μg. iodoacetic acid	382
,, ,, + 200 μg. dichlorodiethylsulphone	367

system ; they cannot therefore inactivate the adenine flavine dinucleotide component.

The experiments in this section indicate that the pyruvate oxidase system is selectively poisoned by H at concentrations which do not affect the other parts of the system, though the concentration of H required for this toxic effect is much larger than that of arsenicals and the two relevant sulphones. Since this work was reported Dixon and Needham (1941) have shown that H is toxic to the pyruvate system of *B. coli*, an enzyme obtained by Still (1940) in clear cell-free solutions ; the optimal *p*H for their enzyme was *p*H 6.2, differing from that of the brain experiments, *p*H 7.3.

Dehydrogenase experiments.—So far the experiments described have been upon partly organized tissue systems, which is reasonable when the effect upon the living cell is being especially considered. But it was desirable to know how the isolated dehydrogenases behaved. Many experiments were therefore done upon the actions of several of these compounds upon standard dehydrogenase preparations ; only representative experiments are given in Table XVI for the 3 substances H, HO_2, and divinylsulphone. In muscle preparations H had no appreciable effect upon succinate, malate, or lactate dehydrogenase ; with brain tissue H had a slight and variable effect on succinodehydrogenase, but the action of H upon pyruvate dehydrogenase was always greater. Nevertheless, as with the arsenicals, the action of H upon the pyruvate dehydrogenase is less than upon the total oxidase, suggesting that there is a factor in the aerobic system, perhaps the phosphokinase (Dixon and Needham, 1946), which is more sensitive.

The toxic action of HO_2 and of divinylsulphone was greater upon the pyruvate dehydrogenase than upon the other dehydrogenases ; on the former the effect of comparatively low concentrations was considerable, which is in agreement with the investigation upon the total oxidase.

Discussion

HO_2, divinylsulphone, and H show a selective action upon the component of the pyruvate system dealing specifically with pyruvate ; after completion of these experiments R. van Heyningen (1941) and workers in the U.S. considerably extended the number of enzymes tried ; very few were sensitive to H, hexokinase (Dixon and Needham, 1946 ; van Heyningen, 1941) being an outstanding exception. Like the arsenicals, HO_2 and divinylsulphone appear to be combining with an -SH group ; this is consistent not only with the toxicity values but

TABLE XVI

EFFECT OF DICHLORODIETHYLSULPHONE (HO_2), DIVINYLSULPHONE (DVS), AND MUSTARD GAS (H) UPON PYRUVATE AND SOME OTHER DEHYDROGENASES. PYRUVATE, 0.018*M*; Na SUCCINATE, 0.082*M*; MALATE, 0.0585*M*; LACTATE, 0.069*M*; BOTH THE LATTER HAD COZYMASE ADDED; TOTAL VOLUME 1.5 ML.

Exp.	Source and type	Incubation time (min.) for poison	Substrate	Decolorization time (in min.) for methylene blue			
				Substrate alone	HO_2 100 μg.	HO_2 200 μg.	DVS 25 μg.
1	Brain	40	Pyruvate	15	40	140	—
			Succinate	13½	24	22	—
2	Brain	40	Pyruvate	16	37½	70	—
			Lactate	6½	9	10	—
3	Brain	40	Pyruvate	23	35	—	40
			Succinate	15	17	—	17½
4	Brain	40	Pyruvate	12½	—	—	a (100 μg.)
5	Brain	40	Pyruvate	17	—	—	80 (50 μg.)
6	Muscle	0	Malate	10	—	10+	—
		0	Succinate	19	—	—	19 (240 μg.)
7	Muscle	40	Malate	7½	—	—	7½
					H (800 μg.)		
8	Brain	20	Pyruvate	13½	22½	200 μg. HO_2 = 0.70 m*M*	
			Succinate	11¼	12½		
9	Brain	40	Pyruvate	28	42	25 μg. DVS = 0.141 m*M*	
			Succinate	28	30		
10	Muscle	20	Succinate	9½	9½	800 μg. H = 3.28 m*M*	
11	Muscle	15	Malate	50	50		
			Succinate	20	22	emulsified with lecithin	
12	Muscle	40	Malate	4	4		
				9	9½		

also with the earlier work (Peters and Walker, 1924–5) on the abolition of -SH reactions in skin slices, etc., by some of these substances, and with other facts known now about the action of the sulphones (Ford-Moore and Lidstone, 1940; Banks, Boursnell, Francis, Hopwood, and Wormall, 1946; Stahmann, Golumbic, Stein, and Fruton, 1946); on the other hand, the idea that H combines as well with the -SH groups in the proteins as it does with thiol groups in compounds like glutathione must be abandoned. In the earlier work (1924–5) it had been found that H, unlike its sulphone when free from dithio compounds, did not abolish the nitroprusside reaction of the fixed -SH groups in tissues; this was set aside at the start of the war, erroneously as it turned out. Other workers (van Heyningen, 1941; Bailey and Webb, 1944; Banks *et al.*, 1946) as well as ourselves (Peters and Wakelin, 1947) have concluded that in proteins the attack of H is only partly upon the -SH groups and that it combines with other groups like COOH. In view of this, it cannot yet be decided how H attacks the pyruvate oxidase system. In another communication (Peters and Wakelin, 1947) the suggestion was made that potentiation by diethyl dithiocarbamate directs the attack of H to the -SH groups. In this relation, with work upon lacrimators, vesicants, and -SH groups, the work of Z. Bacq and his colleagues (for summary see Z. Bacq, 1946) is of interest.

In regard to the toxicity of HO_2 and of the arsenicals to the pyruvate oxidase system, it is interesting to note that Fell and Allsopp (1939, 1946) have found for the lethal doses to tissue culture preparations, 11–18 μM for lewisite oxide and 75 μM for HO_2; these amounts are surprisingly close to those which inactivate this enzyme. If it is not the pyruvate system which is attacked, it must be some as yet unknown enzyme of equal sensitivity.

Note: Since completion of this report, it has been concluded that the pyruvate oxidase system as studied here includes the tricarboxylic acid cycle (Coxon, Liébecq, and Peters; see Peters, Dixon lecture, 1948).

SUMMARY

1. The toxicity of several non-arsenical vesicants and related substances to the pyruvate oxidase system in pigeon brain has been determined.

2. The most toxic substances were dichlorodiethylsulphone (HO_2) and divinylsulphone; it is

suggested that these compounds attack an -SH group.

3. Mustard gas (H) was less toxic ; but with the help of emulsification with lecithin it could serve as a test for antidotes. There is evidence that it is not attacking the -SH group alone.

4. All three substances attacked the pyruvate system selectively, and the pyruvate components of the total oxidase system ; the toxic action upon succinic dehydrogenase, malate dehydrogenase, and lactate dehydrogenase was much less marked, and no toxic action upon amino-acid oxidase was observed with the concentrations used.

We wish to acknowledge the help of members of the team during this work, which was carried out during the war for the Ministry of Supply. We are grateful to the Chief Scientific Officer for permission to publish, to Dr. Ing and Mr. Philpot for diethanoldithiocarbamate, and to Prof. Keilin for preparations of cytochrome C and succinodehydrogenase.

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THE VASODEPRESSOR ACTION OF NORADRENALINE

BY

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In their classical study of the relation between chemical structure of amines and their sympathomimetic actions, Barger and Dale (1910) noted that a dose of ergotoxine sufficient to reverse the pressor effect of *dl*-adrenaline in the spinal cat did not reverse that of *dl*-noradrenaline. The pressor response of the latter substance was greater than that of the former, but relaxation of the isolated non-pregnant uterus was slight. Since that time many workers (Tainter, 1931; Greer, Pinkston, Baxter, and Brannon, 1937, 1938; Gaddum and Goodwin, 1947) have confirmed these findings, and found that yohimbine and most ergot alkaloids in addition to ergotoxine depress but do not reverse the pressor response to noradrenaline. Naturally occurring *l*-adrenaline and synthetic *dl*-noradrenaline were used for purposes of comparison. On some occasions, however, the action of noradrenaline was wholly inhibited or even slightly reversed by ergotamine, though never to the same extent as that of adrenaline (Stehle and Ellsworth, 1937). Melville (1937) showed that certain dioxane derivatives such as 933F and 883F failed to reverse the pressor action of noradrenaline but did reverse that of adrenaline. Dibenamine (Nickerson and Goodman, 1947) has also been used as an adrenaline antagonist.

The difference in response of similar doses of the two amines on the blood pressure of cats and dogs after the administration of an antagonist has been used (*a*) to support the theory that the substance liberated on stimulation of the hepatic nerves is noradrenaline or some similar substance (for references see West, 1947b), and (*b*) to confirm the observation that extracts of various mammalian organs (except placenta) contain a pressor substance with properties like those of noradrenaline (Euler, 1946). It has now been possible to demonstrate the vasodepressor action of noradrenaline in the cat by using large doses. The successful resolution of arterenol (*dl*-noradrenaline) by Tainter, Tullar, and Luduena (1948) has enabled this work to be completed with both *dl*- and *l*-noradrenaline.

METHODS

In different experiments, spinal cats, cats anaesthetized with chloralose or urethane, and rabbits anaesthetized with urethane were used. Blood pressure records were taken from the carotid artery; injections of the drugs were made into the femoral, jugular, or splenic veins, or into the external iliac artery so that the injected solution passed into the vessels of the opposite leg. In some cats, injections were also made into one of the two main splenic arteries. Movements of the duodenum and uterus were recorded directly. The adrenaline antagonists used were ergotoxine (5 mg./kg.), ergotamine tartrate (2 mg./kg.), and dibenamine (15 mg./kg.). For enhancement of the responses, cocaine hydrochloride (8 mg./kg.) was given intravenously.

In one experiment, samples of heparinized blood were taken from the femoral artery, and after being rapidly cooled were subjected to biological analyses (West, 1947b). Dialysed samples were tested on the perfused blood vessels of the frog (West, 1947a), whilst undialysed plasma samples were tested on the isolated uterus and ileum of the rat (de Jalon, Bayo, and de Jalon, 1945). Solutions of *l*-adrenaline, *dl*- and *l*-noradrenaline were prepared from the pure substances, which were kindly supplied by Dr. M. L. Tainter.

RESULTS

During the comparison of the pressor actions of adrenaline and *dl*-noradrenaline in the cat, it was confirmed that ergotoxine and ergotamine antagonized the vasopressor action of adrenaline, so that the vasodilator component was unmasked. No typical reversal occurred with noradrenaline since it has little vasodilator activity in comparable doses. When, however, the dose of noradrenaline was considerably increased (to more than twenty times the corresponding reversal dose of adrenaline), the vasodepressor action of noradrenaline became apparent (Fig. 1). After double vagotomy, the fall of blood pressure following the injection of 250 μg. noradrenaline into the femoral vein was still present. Intravenous atropine likewise was without effect on this response. It is worthy of note that the fall of blood pressure was prolonged, in contrast to

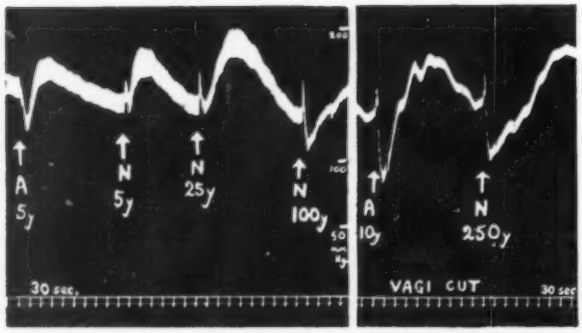

FIG. 1.—Cat 2.5 kg. Chloralose. Ergotoxine. The effect on the blood pressure of doses of *l*-adrenaline (A) and *dl*-noradrenaline (N) before and after vagotomy. All injections into the femoral vein.

the large transient fall after the administration of adrenaline, and never reached the maximum depression shown by doses of adrenaline. These two facts suggest a definite difference in the mode of production of the vasodepressor action.

In cats under chloralose receiving cocaine and ergotamine intravenously, similar reversals occurred, and in addition relatively small doses of noradrenaline produced falls of blood pressure. In one animal which had a steady blood pressure of about 200 mm. Hg throughout the whole experiment, 10 μg. of adrenaline by jugular vein produced maximal vasodilatation, whereas 250 μg. of noradrenaline exerted the maximal effect for this drug. These results were independent of the anaesthetic, since comparable results were obtained in spinal cats and in cats under urethane and chloralose. In a spinal cat, given cocaine and ergotoxine, the vasodepressor action of large doses of noradrenaline was only shown when the blood pressure had been raised to about 100 mm. Hg. As with the adrenaline vasodilatation, there is a limit to the amount of vasodepression obtainable by these drugs.

In a cat under urethane (Fig. 2), 30 μg. *dl*-noradrenaline after dibenamine produced a fall of blood pressure and relaxation of the ileum *in vivo* comparable with that produced by 1 μg. adrenaline. In this experiment, samples of heparinized blood were taken from the femoral artery before the administration of noradrenaline and at the peak of the fall of blood pressure after its administration. Half the number of samples were cooled immediately, centrifuged, and the plasma tested on the isolated uterus and ileum of the rat; the other half were cooled immediately, dialysed against $N/100$ HCl containing 0.1 g. glycine per 100 ml., and the dialysate tested on the perfused blood vessels of the frog. The results of the assays were calculated as noradrenaline and as adrenaline, and indicated that noradrenaline could be detected in the blood in

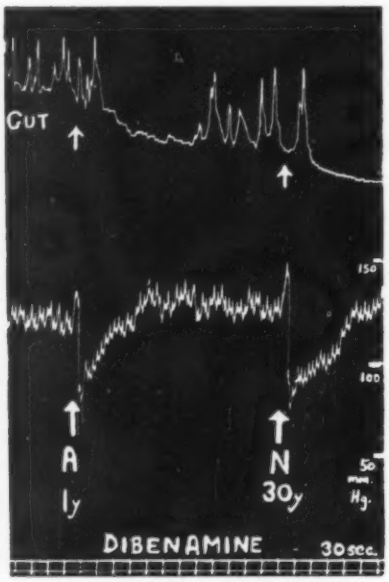

FIG. 2.—Cat 2.0 kg. Urethane. Dibenamine. Relaxation of the gut and fall in blood pressure produced by 1 μg. adrenaline (A) and 30 μg. noradrenaline (N) intrafemorally.

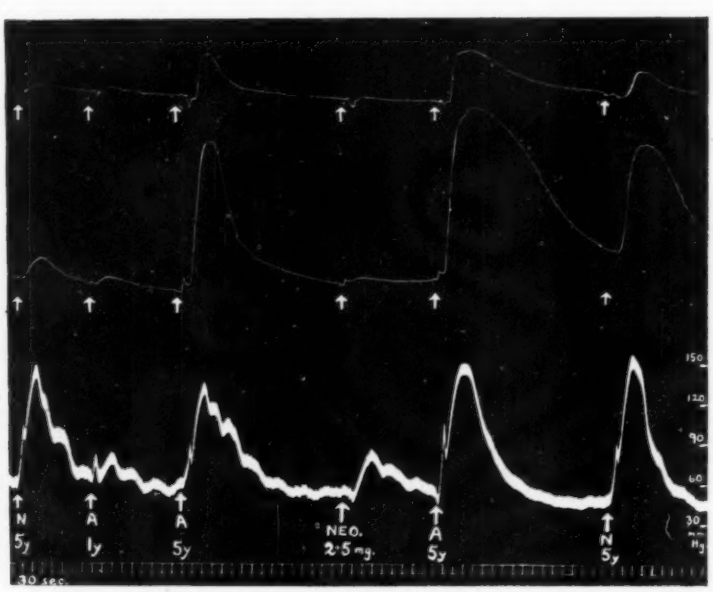

FIG. 3.—Cat 2.5 kg. Chloralose. Top record, right nictitating membrane; middle, left denervated nictitating membrane; bottom, blood pressure. Potentiation of the *l*-adrenaline (A) and *dl*-noradrenaline (N) response by the intravenous injection of 2.5 mg. neoantergan (Neo).

a concentration of 1.0–1.5 μg./ml. after the intravenous administration of 30 μg. It is possible that a small quantity of noradrenaline may be methylated, and that the subsequent fall of blood pressure may be due to adrenaline; this large dose of noradrenaline, on the other hand, may liberate small amounts of adrenaline from other tissues. The tests on the samples of blood indicated, however, that most of the noradrenaline was present as such; adrenaline (0.23–0.50 μg./ml.) was detected in the blood before the injections of the amines.

Effect of antihistamine substances on the vasodepressor response

It is well known that the motor actions of adrenaline are potentiated by antihistamine substances, presumably by antagonism of the histamine liberated during the adrenaline response. It seemed of interest, therefore, to determine the effect of antihistamine substances on the noradrenaline response, before and after ergotoxine or dibenamine. Neoantergan intravenously (1 mg./kg.) potentiated both the *l*-adrenaline and the *dl*-noradrenaline actions on the blood pressure and nictitating membranes (one denervated) of a cat under chloralose (Fig. 3). Benadryl and antistine in suitable doses potentiated these actions in a similar manner. After ergotoxine, however, the depressor responses to 5 μg. adrenaline and 100 μg. noradrenaline were unaffected. This indicates that the vasodepressor response to large doses of noradrenaline after ergotoxine or dibenamine is not due to a liberation of histamine.

Reversal of the vasodepressor response

Recently Neil, Redwood, and Schweitzer (1948) reported that the depressor response to aortic or sinus nerve stimulation in cats under pentobarbitone was converted into a rise of blood pressure by the intravenous injection of chloralose. The exact mechanism by which this change occurred was not determined. Small amounts of pentobarbitone were therefore administered to cats under chloralose. In Fig. 4, cocaine and dibenamine were first given intravenously, followed by vagotomy and neoantergan administration. Doses of 1 μg. adrenaline and 30 μg. noradrenaline produced the typical falls in blood pressure and relaxation of the non-pregnant uterus. Pentobarbitone (7 mg.) was then given and the cat was left for one hour. After this time, the vasodepressor response to large doses of noradrenaline was converted to a rise of blood pressure. The normal reversed action of adrenaline was unaffected, as also was the response to small doses of *iso*propyl-noradrenaline (Fig. 4, Is). After some time spontaneous reversion of the pressor to the depressor response with noradrenaline occurred. In order to exclude the possible effects of altered pulmonary ventilation on the blood pressure, this experiment was repeated with similar results under artificial respiration with bilateral open pneumothorax.

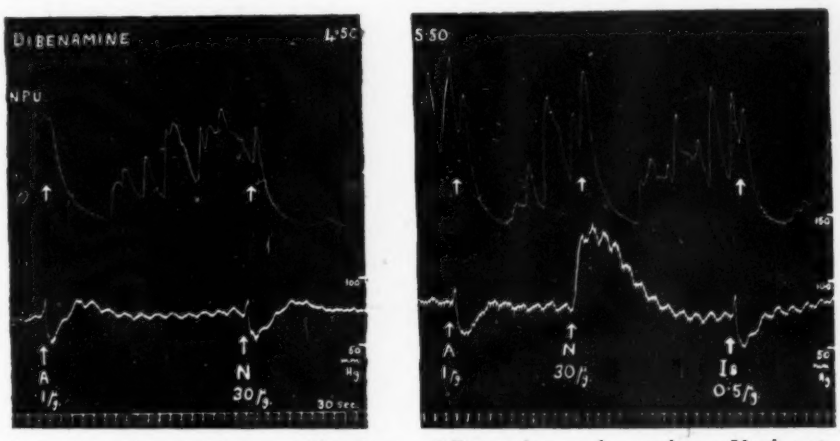

FIG. 4.—Cat 1.8 kg. Chloralose. Dibenamine and cocaine. Vagi cut. Neoantergan 1 mg./kg. intravenously. Relaxation of the non-pregnant uterus and fall in blood pressure produced by intrajugular doses of adrenaline (A) and noradrenaline (N). Between the left and right records, a period of one hour during which time 7 mg. pentobarbitone were given intravenously. Note the reversal of the noradrenaline action on the blood pressure but not that of adrenaline. The response to 0.5 μg. *iso*propyl-noradrenaline (Is) was also unaffected.

Injections by other routes

The more active *l*-noradrenaline was used for these experiments. In cats under chloralose, it was confirmed that the ratio of equipressor doses of *l*-noradrenaline and *l*-adrenaline was about 0.6 (Tainter, Tullar, and Luduena, 1948; Graham, 1949). After the ergot alkaloids or dibenamine, both substances exhibited depressor responses by the femoral, jugular, and intraportal routes, but the ratio of doses producing maximal vasodepression was reduced from 30:1 (Fig. 2) to 8:1 (Fig. 5, FV) for the femoral route. When injected into the artery supplying the caudal end of the spleen of cats without ergot or dibenamine, noradrenaline produced pure rises of blood pressure at all dose levels, whereas adrenaline injections gave small rises of pressure followed by large falls. After dibenamine, similar injections of both drugs produced depressor responses, that of noradrenaline being much less marked than that of

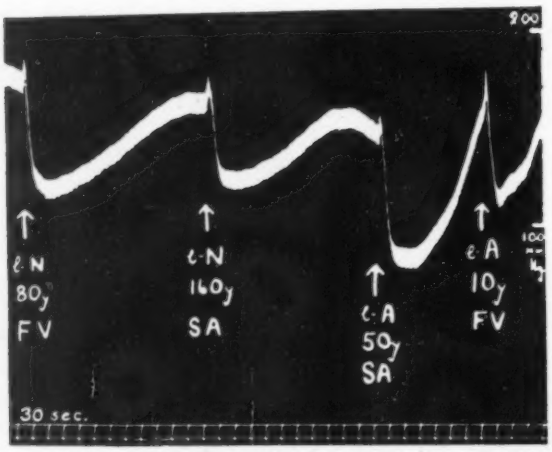

FIG. 5.—Cat 3.2 kg. Chloralose. Ergotoxine. The effect on the blood pressure of doses of *l*-adrenaline (A) and *l*-noradrenaline (N) injected into the splenic artery (SA) and the femoral vein (FV).

adrenaline (Fig. 5, SA). These effects were independent of the anaesthetic, since comparable results were obtained in cats under urethane or ether. As reported earlier (West, 1948), the latent period of noradrenaline injections by splenic artery was always longer than that of adrenaline.

When injected into the external iliac artery (so that the injected solution passed into the vessels of the opposite leg), 1 μg. adrenaline after cocaine and dibenamine was required to cause the same fall of blood pressure as 0.5 μg. adrenaline by jugular vein. When noradrenaline was injected in a similar manner, 15 μg. by the jugular vein produced a comparable fall of blood pressure, but all intra-arterial doses up to 100 μg. failed to elicit the true vasodepressor response. Further evidence that the vasodepressor responses of adrenaline and noradrenaline are the result of different mechanisms was shown by perfusion experiments. The hind limbs of cats were perfused with oxygenated Locke's solution at 37° C., and the outflows recorded in a drop-timer (Gaddum and Kwiatkowski, 1938). In this preparation, small doses of adrenaline caused vasodilatation after dibenamine (given slowly), whereas similar doses of noradrenaline were without effect. In addition, an increase in the dose of the latter had no vasodepressor action.

Experiments with rabbits

In a few rabbits, it was shown that the ratio of equi-pressor doses of *l*-noradrenaline and *l*-adrenaline was about 2.0, confirming previous observations (West, 1948) that adrenaline is more active than noradrenaline in this animal. After ergotoxine, pressor responses of both drugs were almost wholly reduced but not reversed. Cannon and Lyman (1913) reported that the rabbit, in contrast to the cat, appears to lack a sympathetic depressor component capable of being unmasked by blocking agents such as ergotoxine. Large doses of *l*-adrenaline and *l*-noradrenaline (up to 1 mg.) failed to elicit any depressor action. Similar results were found in rabbits after dibenamine, though it was difficult to administer this drug satisfactorily to these animals.

DISCUSSION

Previous workers have shown that a dose of a sympatholytic agent sufficient to reverse the pressor effect of adrenaline does not reverse that of noradrenaline. Equi-pressor doses of the two amines before the administration of the antagonist were used to demonstrate this difference in response. The observation that noradrenaline in suitable doses can produce regular vasodepressor responses in cats which have been treated with ergotoxine or dibenamine is important. The fall in blood pressure only occurs after large doses of noradrenaline; also it is always more prolonged than that produced by adrenaline, and never reaches such low levels. West (1948) showed that after large intraportal doses of dihydroergotamine in cats, it was sometimes possible to obtain depressor responses to small intraportal doses of noradrenaline, when similar doses of adrenaline were without effect. Nickerson and Goodman (1947) reported that the reversal of the adrenergic vasopressor effect by dibenamine was not altered by atropine, benadryl, and pyribenzamine, or by the anaesthetic used, and was due to vasodilatation.

The mechanism by which the vasodepressor response to noradrenaline is produced is not clear. Several possibilities exist: (1) Constriction of the coronary arteries may occur. Bacq and Fischer (1947) reported that extracts of human coronary nerves and arteries contain adrenaline, and they suggested that in certain diseases the synthesis of adrenaline is stopped at the stage of noradrenaline, which may have a vasoconstrictor action on the coronary vessels. In direct contrast to this suggestion, Marsh, Pelletier, and Ross (1948) showed that noradrenaline increased cardiac output and coronary flow in isolated mammalian hearts. (2) Constriction of branches of the portal vein in the liver may be produced. In two cats given dibenamine, the whole portal vein was closed, and when the blood pressure was stabilized large doses of noradrenaline still exhibited vasodepressor responses. (3) Constriction of the pulmonary arteries may take place. A rise in pressure in the pulmonary vessels has been shown to induce a fall in the systemic circulation in a cat (Parin, 1947); this fall was the result of a cardiac component and a circulatory component. The changes in the general circulation were due to vasodilatation, the spleen increasing in volume as long as its nerve supply remained intact. It was considered probable that pressoreceptor reflexes arise in the pulmonary artery, and it is possible that the depressor response to noradrenaline may be produced by this mechanism. In all the experiments with pentobarbitone, reversal to a pressor response did not occur immediately but was maximal after about an hour, when the vasodilator action of adrenaline still existed. It is not known whether excess of pentobarbitone affects the fall in systemic pressure induced by a rise in pressure in the pulmonary arteries.

Summary

1. Large doses of *l*- and *dl*-noradrenaline produced vasodepressor responses in cats given ergotoxine or dibenamine. No such action was noted in rabbits.

2. The mechanism by which this fall of blood pressure is produced has been studied. It is probably not due to vasodilatation, as is the normal adrenaline reversal response.

3. Intravenous injection of pentobarbitone converted the depressor response to a rise of blood pressure in cats under chloralose.

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Brit. J. Pharmacol. (1949), **4**, 68.

MIRACIL. CLINICAL TRIAL ON PATIENTS INFECTED WITH *SCHISTOSOMA HAEMATOBIUM* AND *S. MANSONI*

BY

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A previous paper (Hawking and Ross, 1948) has described the toxicology, absorption, and excretion of miracil D in monkeys and in healthy volunteers. The present paper records a clinical trial of this compound on patients infected with *Schistosoma haematobium* and/or *S. mansoni*. A preliminary note on these findings was published by Blair, Hawking, and Ross (1947).

Miracil D is the hydrochloride of 1-methyl-4-β-diethylaminoethylaminothioxanthone:

It was synthesized by Mauss, and shown by Kikuth, Gönnert, and Mauss (1946) and Kikuth and Gönnert (1948) to have considerable therapeutic activity for mice and monkeys infected with *S. mansoni*. The compound is administered by mouth. Subcutaneous or intramuscular injection causes considerable local irritation, and when given intravenously the toxicity is much greater than when given orally.

As shown in the previous paper, it is rapidly absorbed from the alimentary canal, and 2¼ hours after a single dose of 0.2 g. to an average man the concentration in the blood rises to about 1 mg. per litre. Only about 7 per cent of the drug is excreted in the urine, and little appears in the faeces; presumably about 90 per cent is degraded in the body. There is little tendency for the drug to accumulate. Deliberate prolonged overdosage in animals produced degenerative changes in the liver and the renal tubules in a few animals, but these were usually much less than would have been expected.

Organization of therapeutic trial

The present investigation was carried out at Salisbury, Southern Rhodesia, beginning in the second half of 1947. Patients with active schistosome infections were selected for the trials. Urines and stools were collected and examined for the presence of eggs and miracidia. The urines were examined by collecting the terminal specimen of urine, centrifuging it, and examining the resultant deposit for eggs. Originally the faeces were examined by collecting material from the outer layers of the stool together with any blood or mucus present and making an emulsion, with pond water, in a 3-in. × 1-in. tube. This emulsion was then filtered through a coffee strainer and transfered to a conical urine glass, further pond water was added, and the whole was allowed to settle. The supernatant was poured off, fresh water was added, and this washing process was repeated several times. Finally the resultant deposit was examined microscopically for the presence of eggs. Later, improved methods of diagnosis were used both for urine and faeces, depending on the hatching out of miracidia from the washed deposit and their identification with a hand-lens under indirect illumination (Gorman, Meeser, Ross, and Blair, 1947; Meeser, Ross, and Blair, 1948). Most of the therapeutic trials were made on young patients who were passing eggs in large numbers; infections in adults are less satisfactory for this purpose, since excretion of eggs is often intermittent.

Previous to treatment each patient was examined clinically and weighed stripped. The age in Africans can be ascertained only approximately. Before commencing treatment a haemoglobin estimation was

made using a Newcomer standard (acid haematin) measured with a Klett-Bio colorimeter; a white blood corpuscle count and a differential leucocyte count were also made. The drug was given as uncoated 100-mg. tablets (kindly made up by Messrs. Burroughs Wellcome and Co.) followed by a drink of water. With patients receiving once-daily doses it was usually given at 2–3 p.m., about two hours after the midday meal, but occasionally it was given at 10 a.m. Doses were not given on Sundays. All doses were given under the personal supervision of one of the writers.

After treatment the patients were followed up at 7-day intervals for at least sixteen weeks. In some of the groups these weekly examinations were interrupted for four weeks by absence during the school holidays. The successful follow-up of so many of the school-children was greatly assisted by the helpful co-operation of Messrs. C. M. Drury and F. G. Loveridge, successive headmasters of the Salisbury African School. At each follow-up a specimen of urine and/or stool was examined by the methods already described. Patients were considered "negative" if there were no eggs, or only calcified eggs were present. Patients were classified according to whether (1) active miracidia could be hatched (A), (2) living eggs were found but no miracidia hatched (E), (3) only dead eggs were present (D), (4) no eggs could be detected (O). A haemoglobin estimation and a white cell count were also made. If the white cell count was altered significantly from the previous count, then a differential cell count was done to obviate the insidious onset of agranulocytosis or any other type of cell change.

Clinical Results

The patients treated may be divided into five series, some of which may be subdivided into groups.

First series of patients

The patients of the first series were divided into five groups. The first group consisted of three adult male Africans employed in the laboratory; their ages were 30–45 years. The second group was made up of ten male African school-boys; their ages ranged from 14–17 years, average 16 years, and their weights ranged from 40 to 64 kg., average 53 kg. One boy in this group, who felt unwell on the fourth day, stopped treatment after one dose of 50 mg. and three doses of 100 mg. The third group consisted of thirteen male African school-boys; their ages ranged from 14–17 years, average 15½ years, and their weights from 33 to 66 kg., average 54 kg. The fourth group consisted of seventeen Eurafrican (coloured) school-children, thirteen males and four females, whose ages ranged from 10–17 years, with an average of 13 years. As there were considerable differences in weight in this group, a demarcating line of 40 kg. was taken, those over 40 kg. (six boys weighing 41 to 68 kg., average 49 kg.) forming one sub-group, while those less than 40 kg. (seven boys and four girls weighing 30 to 40 kg., average 35 kg.) formed another. The fifth group consisted of one male European, aged 17 years.

Of the forty-four patients under treatment, in the first group two had *S. haematobium* infections and one *S. mansoni*; in the second group, five had *S. haematobium* infections and five had double infections of *S. haematobium* and *S. mansoni*; in the third group, two had *S. mansoni* infections, nine had *S. haematobium* infections, and two had double infections; in the fourth group, fifteen had *S. haematobium* infections and two had double infections; and in the fifth group there was a single *S. haematobium* infection.

The results of treating the first series of patients are shown in Table I.

It must be remembered that in schistosomiasis egg production as discovered by examination of the excreta may frequently be intermittent. During a series of repeat examinations of the same patient negative findings are not uncommon. This may be due to a number of factors, such as ageing, relatively infertile worms ceasing to produce eggs regularly, or advanced damage of bladder and bowel with consequent fibrosis which makes the passage of eggs more difficult. Thus in Group III one case was positive, negative, and then positive, and a second case negative, positive, and again positive at the end of treatment, four weeks later and eight weeks later respectively. Furthermore, although reinfection during the time of the follow-up was unlikely, it was by no means impossible, and after eight weeks cases classified as "relapses" may possibly have been reinfections. These factors make the assessment of cure at any one time difficult. From Table I it can be seen that 12 infections (1 of *S. mansoni* and 11 of *S. haematobium*) were apparently cured—i.e., no miracidia or fresh eggs were found twelve weeks after treatment; but in 9 of these 12 infections the excretion of eggs had initially been light or irregular. The number of patients cured was smaller than the number of infections cured, since 9 patients initially had double infections, and one species might disappear while the other remained. The results of treating these double infections were as follows; among the eight patients followed for twelve weeks, not one remained negative for both infections although three became negative for *S. haematobium* alone and one for *S. mansoni* alone.

TABLE I

SUMMARY OF THE EFFECTS OF TREATMENT BY MIRACIL D (1ST SERIES)

H indicates *S. haematobium*, and M indicates *S. mansoni* infections

Group	No. of patients	Average weight kg.	Doses mg.	No. of patients passing no living ova/No. treated							
				End of treatment		After 4 weeks		After 8 weeks		After 12 weeks	
				H	M	H	M	H	M	H	M
I	3 (2H, 1M)	56.5	50 × 1 100 × 1 150 × 1 200 × 13	2/2	0/1	2/2	0/1	2/2	0/1	2/2	0/1
II	10 (10H, 5M)	53	50 × 1 100 × 13a 300 × 12	1/10 (6/9) *	1/5 (1/4)	3/9 (3/8)	3/4 (3/3)	1/9 (1/8)	2/4 (1/3)	2/9 (3/8)	1/4 (1/3)
III	13 (11H, 4M)	54	50 × 1 200 × 18–22	1/11 (7/9)	1/4 (0/4)	2/11 (2/9)	1/4 (1/4)	3/11 (4/9)	0/4 (0/4)	4/11 (3/9)	0/4 (0/4)
IV	11 (11H, 2M)	35	50 × 1 100 × 6 200 × 13	1/17 (6/16)	0/2 (0/1)	3/17 (3/16)	0/2 (0/1)	3/17 (3/16)	0/2 (0/1)	3/17 (4/16)	0/2 (0/1)
	6 (6H)	49	50 × 1 200 × 19								
V	1 (1H)	59	50 × 1 200 × 18	1/1		1/1		0/1		0/1	
Totals	44 (41H, 12M)			6/41 (19/34)	2/12 (1/9)	11/40 (8/33)	4/11 (4/8) b	9/40 (8/33) b	2/11 (1/8) b	11/40 (10/33) b	1/11 (1/8)

(a) 1 boy infected with *S. haematobium* received only 1 dose of 50 mg. and 3 doses of 100 mg.
(b) 1 patient with a double infection could not be followed up.

*Brackets refer to cases from which hatched miracidia could be obtained.

TABLE II

SUMMARY OF THE EFFECTS OF TREATMENT (2ND SERIES)

Boys given up to 11 daily doses of 0.6 g. during 15 days

A = Miracidia hatched and active. E = Living eggs. D = Dead eggs. O = No eggs or miracidia. M = *S. mansoni*

No.	Age	Total dose		Initial state			Excretion of ova at number of weeks after end of treatment											
		g.	mg./kg.	RBC	Ova	Miracidia	1	2	3	4	5	6	7	8	9	14	15	16
1	13	3.0	77	++	+++	++	E	E	O	D	O	O	O	O	O	O	O	O
2	15	3.6	83	+++	++	+	A	E	A	D	A	A	E	A	A	A	A	A
3	17	3.0	46	+++	++	±	—	O	E	A	E	A	A	O	A	—	—	A
Also *mansoni*						AM	AM				AM			AM				AM
4	15	6.6	150	++	+++	++	D	O	O	O	E	O	O	O	O	O	D	O
5	18	2.4	35	+	+	+	A	D	O	O	O	O	O	O	O	O	O	A
6	17	3.6	61	+++	+++	++++	E	O	O	O	O	O	A	A	A	D	E	O
7	15	3.0	54	++	+++	++	O	D	E	D	O	A	A	A	A	A	A	A
8	15	4.2	91	++++	+++	+++	A	A	A	A	A	A	A	A	A	A	A	A
9	13	4.2	98	+++	++	++	A	A	O	D	O	O	O	O	O	D	O	D
10	14	3.6	84	++++	++++	++++	A	E	O	D	A	D	A	A	A	A	E	A
11	16	3.0	55	++++	++++	+++	A	E	D	D	O	D	O	O	D	O	O	O
12	15	1.8	35	++++	+++	+++	A	A	A	A	A	E	A	O	A	E	E	D
13	15	3.6	69	+++	+++	+++	A	E	—	O	O	E	A	A	A	A	E	A
Proportion not passing living eggs (*haematobium*)							—	6/13	7/13	10/13	7/13	6/13	6/13	6/13	6/13	6/13	5/13	4/13

TABLE III

SUMMARY OF THE EFFECTS OF TREATMENT (3RD SERIES)

Children given 0.3, 0.4, 0.5, 0.6, 0, 0.6, 0.7, 0.7, 0.6, 0.6 g. on successive days (per 45 to 55 kg.). All males except Nos. 16, 17, and 18. Symbols as in Table II

No.	Age	Total dose		Initial state March 31 1948			Excretion of ova at number of weeks after end of treatment													
		g.	mg./kg.	RBC	Ova	Miracidia	1*	2	3	4	5	6	7	8	9	10	11	12	13	14	15
14	15	5.0	104	+++	++	++	A	E	D	D	O	O	D	O	O	O	O	O	O	D	—
15	19	6.0	104	++	++	++	O	O	O	O	O	O	O	O	O	O	O	A	O	A	A
16	13	4.0	90	+	++	++	A					O	O	O	O	O	O	O	O	O	O
17	14	4.0	90	+++	+	+	E					O	O	O	O	O	O	O	O	O	O
18	14	3.5	90	+	+	+	O					O	D	O	O	O	O	O	D	O	O
19	18	5.0	100	++	+	+	E					O	—	O	O	O	O	O	A	A	O
20	15	5.0	93	+++	++	++	A					A	A	E	A	A	A	A	A	A	O
21	14	4.0	97	++++	+++	+++	E					A	O	A	—	E	O	O	O	O	O
22	14	4.0	92	++	++	+	A					O	O	O	D	O	O	O	O	A	O
23	14	4.0	92	++	++	+	A					—	O	—	O	O	O	O	O	O	O
24	15	5.0	98	+	+	+	E					O	O	O	O	O	O	O	O	O	O
25	14	4.4	93	++	++	+	A					O	—	O	D	O	O	O	O	O	O
26	14	3.6	103	++	++	++	A					O	O	D	O	O	O	O	D	E	O
27	15	3.8	79	++++	+++	+++	O					—	—	O	D	O	—	O	A	O	A
28	15	6.0	104	+	+	+	E					—	—	—	—	—	—	A	A	A	A
					+M	+	—					—	—	—	—	—	—	A	A	A	A
29	18	5.3	92	+	+	+	A					D	—	O	D	O	O	A	O	E	O
30	19	3.9	64	+	+	+	O					O	O	O	O	O	O	O	O	O	A
31	18	2.5	40	++	++	++	A					O	O	O	D	O	O	O	O	O	—
Proportion not passing living ova (*haematobium*)							4/18					13/15	11/13	14/16	15/17	15/17	14/18	13/18	13/18	12/18	12/18

* 3 days after last dose

If the results are reckoned in terms of individual patients, and the type of infection is disregarded, then out of forty-three patients treated only eight patients no longer passed eggs twelve weeks after treatment had finished; two of these were in Group I, one in Group II, three in Group III, and two in Group IV. On the other hand, if the cases are judged by the criterion whether or not miracidia could be hatched from the excreta (satisfactory data on this subject are not available for all the patients), it is found that at the end of treatment 19 out of 34 cases of *haematobium* infection had become negative. Two weeks after treatment 15 out of 26 cases were negative; at four weeks after treatment the results by this test (8 negative out of 33) were similar to those judged by the presence or absence of living ova (11 negative out of 40). The results with infections of *S. mansoni* were approximately the same whether judged by the presence of viable eggs or by the hatching of miracidia.

From this series it was concluded that the therapeutic effect of miracil in daily doses of 0.3 g. was not great. However, in view of reports from workers at Cairo that patients could tolerate larger doses of miracil than 0.3 g. daily, and in view of the failure of miracidia to hatch in many of the cases after miracil treatment, further trials were instituted using higher levels of dosage.

Second series of patients

These consisted of thirteen African school-boys, who were given a daily dose of 0.6 g. per day during 15 consecutive days (omitting the 11th, 12th, and 13th days on account of the weekend). Many boys complained of abdominal pain and vomiting (some of which may have been psychological in origin), so that attendance for treatment was irregular and only one of the boys, No. 4, completed the full course. Two of the boys had been treated with miracil six months earlier as part of the first series, No. 2 having received 0.35 g. and No. 5 having received 4.25 g. without any apparent benefit. The details concerning these patients together with the results of treatment are shown in Table II.

In spite of the great irregularity of the dosage, the therapeutic effects of the compound are much more definite than those observed in the first trial. The effects are most marked at three weeks after the end of treatment, at which time 10 out of the 13 boys had ceased to pass living eggs. After this some of the boys began again to show signs of infection. For the purpose of this paper, these cases are interpreted as relapses, although the possibility that some were reinfections cannot be excluded. By the sixteenth week 4 out of the 13 were still free from living eggs; these 4 boys had presumably been cured—i.e., sterilized by the treatment. The therapeutic effect was not in direct proportion to the dose. The boys who were cured had received total doses of 77, 150, 98, and 55 mg. per kg. In other boys—e.g., No. 2 and No. 8—total doses of 83 and 91 mg. per kg. had exerted little or no definite effect on the excretion of living eggs.

Third series of patients

In view of the encouraging early results of this second series of patients, a third series was commenced. The patients were again school-children. A dose schedule for subjects of medium weight (45–55 kg.) was planned as follows: 1st day 0.3 g., 2nd day 0.4 g., 3rd day 0.5 g., 4th and 6th days 0.6 g. (5th day omitted), 7th and 8th days 0.7 g., 9th and 10th days 0.6 g., total 5.0 g. Adjustments were made for patients lighter or heavier than this weight-range. These children took the drug much better than those of the previous series did, even though the average daily dose was greater. They actually received total doses ranging from 40 to 104 mg./kg., average 90 mg./kg.; the average daily dose was about 8–10 mg./kg. The details about these patients and the results of treatment are given in Table III. In addition six similar children (five infected with *S. haematobium* and one with *S. mansoni*) chosen for controls were given placebo tablets of sodium citrate dyed yellow so as to resemble miracil; the details of these are given in Table IV for comparison. At 6 weeks after the end of treatment, 13 out of 15 treated patients who were examined no longer passed live eggs; only 2 of those examined still showed signs of an active infection. Fifteen weeks after treatment 9 out of 18 appeared completely free from infection, and 3 others passed only occasional dead eggs. One patient had never responded to treatment, while the remaining five showed a temporary response but the infection reappeared. One patient (No. 28) who was also infected with *S. mansoni* continued to pass ova in the faeces. The six controls (Table IV) continued to pass large numbers of living eggs throughout the period of observation, apart from the fact that a girl (No. 32) with a double infection of *haematobium* and *mansoni* ceased to pass *mansoni* ova in the faeces.

Fourth series—maximum tolerated doses of miracil

In order to discover the maximum amounts of miracil which could be tolerated, larger doses were given to patients with *haematobium* schistosomiasis who had been put in hospital for some other

TABLE IV

SUMMARY OF THE EXCRETION OF OVA IN SIX UNTREATED CONTROL CHILDREN, CHOSEN AT RANDOM FROM THE THIRD SERIES

Symbols as in Table II

No.	Age and sex	Wt. kg.	Initial state			Excretion of ova at number of weeks after end of treatment											
			RBC	Ova	Miracidia	1	2–5	6	7	8	9	10	11	12	13	14	15
32	13♀	37.5	++	+	+	A	—	A	A	O	A	A	A	A	A	E	A
				+M	++	OM			OM		OM		OM		OM		OM
33	17♂	53	+	+	+	A	—	A	A	A	A	A	A	A	A	A	A
34	14♂	46	+	+	++	E	—	O	A	A	A	—	A	A	A	A	A
35	14♂	46	+	+	+	A	—	A	A	A	A	A	A	A	A	A	A
36	15♂	37	+	+	+	A	—	A	A	A	A	A	A	A	A	A	A
37	16♂	43	—	O	+	O	—	O	O	O	O	O	O	O	O	O	O
				+M		AM		—	OM	AM	OM	AM	AM	AM	AM	AM	AM
Proportion not passing living ova						0/6		1/5	1/6	1/6	1/6	0/5	0/6	0/6	0/6	0/6	0/6

reason. As shown in Table V, three patients were given 0.4 g. three times a day for about 9 days (one patient only for 6 days), and three were given 0.6 g. three times daily for 6 days. Only one of these patients, No. 40, showed any toxic effect that might have been due to the drug; he complained of abdominal pain and vomited on the fifth day; the significance of this vomiting is uncertain. Before treatment began, miracidia could be hatched out from the urine of five of these patients. On the last day of their treatments, the urines of two of these five patients were free from eggs; the urines of the other three contained apparently living eggs but the miracidia did not hatch. Another patient, No. 44, was given 0.6 g. twice daily for two days; soon after the first dose on the second morning he vomited. The dose was reduced to 0.4 g. once daily for two days; nausea was felt. On the fifth day he was given 0.3 g. twice and he had nausea and vomited. Four days later the urine still contained eggs but miracidia could not be hatched. Four other patients (Nos. 45–48; not in hospital) were given once-daily doses as shown, which were increased during the five days of treatment to a maximum of 1.6 g. Two of these (46 and 47) had no toxic symptoms at all. One (No. 45) weighing 54 kg. complained of abdominal pain on the 4th and 5th days after doses of 1.2 g. and 1.6 g. respectively; he vomited after the last dose, without bringing up much of the compound. One patient (No. 48; weighing 32 kg.) had abdominal pains, nausea, and vomiting on the 4th and 5th days after doses of 0.8 and 1.0 g. respectively. On the day following treatment miracidia could be hatched from the urines of three out of the four patients. Six of these patients have been followed for 15 weeks; at the end of this period, none passed living ova.

Fifth series of patients

Four miscellaneous patients were treated according to various schedules. The details about these are shown in Table V. In the two European children (Nos. 51 and 52), the skin was stained yellow by the drug. Three of these patients seemed to have been cured; one relapsed (or was re-infected) after 11 weeks.

Toxicity

The patients were questioned daily when given their dose for any toxic effects of the treatment, but with Africans it is generally difficult to evaluate reports of minor symptoms which may be exaggerated or concealed. The symptoms of possible toxicity during this investigation were still more difficult to evaluate because they tended to be more pronounced during the less intense courses than in the more intense ones.

In the first series (treated with 0.2–0.3 g. daily) two boys reported slight or moderate nausea, each on one occasion, but its relation to miracil is uncertain. One fairly old man in Group I suffered more severe symptoms. He began taking miracil on July 21. On Aug. 2 he vomited, and for the next two days there was nausea, lack of appetite, and weakness. On Aug. 4 the drug was stopped (after 12 doses totalling 2.1 g.) but was started again soon after. On Aug. 5 he felt all right; haemoglobin 86 per cent, W.B.C. 4,200 per cu.mm. as at beginning of treatment. On Aug. 7 and 8 nausea, anorexia, and vomiting, but he continued at work these two days. On Aug. 9 he could not come to work; cough, shortness of breath, slight disorientation, pulse rate 96. On Aug. 10 he

TABLE V

SUMMARY OF THE EFFECTS OF TREATMENT (4TH AND 5TH SERIES)

All males except 51 and 52, who were European girls. Symbols as in Table II

No.	Age	Total dose		Schedule, g.	Initial state			Excretion of ova at number of weeks after last dose														Symptoms
		g.	mg./kg.		RBC	Ova	Miracidia	1*	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
								FOURTH SERIES														
38	30	10.4	190	0.4 × 2, 1 day 0.4 × 3, 8 days	++	++	++	O (3)	A	—	A	—	A	O	—	—	—	O	—	—	—	—	None
39	13	10.4	298	0.4 × 2, 1 day 0.4 × 3, 8 days	++	++	+	E (3)	E														None
40	18	6.8	121	0.4 × 3, 6 days	occ.	+ +M	+ +M	E OM (3)	O	—	O	O	O OM	O OM	O OM	O	O	O	O OM	O	O OM	O OM	Pain and vomiting 5th day
41	35	10.8	190	0.6 × 3, 6 days	++	—	—	— (0)															None
42	26	10.8	202	0.6 × 3, 6 days	occ.	+	+	E (0)															None
43	20	10.8	212	0.6 × 3, 6 days	+++	+	—	O (0)	D	D	D	O	O	D	O	O	D	O	O	D	O	D	None
44	25	4.2	74	0.6 × 2, 2 days 0.4 × 1, 2 days 0.3 × 2, 1 day	++	++	++	E (4)	—	—	O	—	O	O	O	O	D	—	O	—	O	O	Nausea and vomiting
45	20	5.8	107	0.6 × 1, 1 day 1.2 × 1, 3 days 1.6 × 1, 1 day	+++	++	++	A (1)	A	E	D	D	O	D	D	D	D	D	—	—	O	O	Abdominal pain 2 days. Vomited last day
46	14	4.6	107	0.6 × 1, 1 day 0.8 × 1, 1 day 1.0 × 1, 2 days 1.2 × 1, 1 day	++	+	+	A (1)	A	A	D	D	D	D									None
47	18	5.8	105	0.6 × 1, 1 day 1.2 × 1, 3 days 1.6 × 1, 1 day	++	+	+	E (1)	E	A	O	O	O	D	O	O	O	O	—	—	O	O	None
48	10	3.6	112	0.4 × 1, 1 day 0.6 × 1, 1 day 0.8 × 1, 2 days 1.0 × 1, 1 day	+++	+++	++	A (1)	A	A	O	O	O	D	O	O	O	D	—	—	D	O	Abdominal pain and nausea 2 days. Slight vomiting 2 days
								FIFTH SERIES														
49	18	6.0	91	0.3 × 2,10 days	++	++	++	O	O	D	D	O	O	O	O	O							
50	7	5.6	154	0.2 × 4, 8 days	++	+	+	A	O	O	O	O	O	D	O	O	O	O	O	O	O	O	
51	8	3.0	97	0.3 × 1, 4 days 0.4 × 1, 3 days 0.3 × 1, 2 days	++	++	+	A	O	—	—	O	—	—	—	—	O	—	O	O	O	O	
52	4	1.8	92	0.2 × 1, 9 days	++	++	++	—	D	D	—	—	—	O	—	O	O	A	A	—	O	D	

*Figures in brackets show days after last dose

began to take food. Aug. 11, all right; chest, nothing abnormal discovered. Aug. 15, haemoglobin 63 per cent, W.B.C. 3,500 per cu.mm. These symptoms may have been due to other causes, although there was no clear evidence of an intercurrent infection, and they cannot be proved to have been due to miracil.

None of the patients treated showed significant changes in the haemoglobin percentage or white cell counts during or after treatment. None complained of tinnitus (Africans might not report it even if present). There were no gross changes in the urine; slight albuminuria or haematuria was masked by the schistosomiasis. The European of the first series, Group V, became distinctly yellow in the skin and conjunctivae while taking the drug, but he had no other symptoms.

In the second series there was a good deal of difficulty in getting the children to take the full doses (0.6 g. once daily); they complained mainly of loss of appetite and abdominal pain and there seems to have been much deliberate absenteeism. The doses were given as 6 tablets each of 100 mg., and this large number of separate pills may have had a bad psychological effect. One boy, No. 4, took 6.6 g. On the last day of his course (Feb. 17) he complained of pain in the abdomen and inability to see clearly. On Feb. 24 he developed an acute generalized pruritus, which was severe during the short time it lasted. The next day he showed a close herpetiform rash on the outstanding margin of his R. trapezius muscle, perhaps an affection of the accessory nerve. In this connexion it may be recalled that Alves and Blair (1946) observed a herpetiform rash over the trapezius muscle in one of 100 Africans treated by their intensive course of sodium antimonyl tartrate. Ritchken and Cantor (1947) noted an association between the administration of a similar intensive course of antimony and the onset of herpes zoster in five of their patients. These cases of herpes and herpetiform rash had been attributed to the antimony, but in view of a similar occurrence during treatment with miracil it is possible that the herpes is really caused by the disintegration products of the dying schistosomes.

In the third series most of the children registered no complaints except loss of appetite; but one child (No. 28) felt ill after the penultimate dose. Another complained of vomiting and of feeling generally weak on the 8th day after beginning treatment, but he took his other doses without incident.

In the fourth series (which received the highest doses) the symptoms of toxicity were restricted to abdominal pain, nausea, and vomiting in some of the patients, and they have already been described. In the fifth series no toxic symptoms were observed.

Concentration of miracil in the blood of patients

Three adult male Africans, who were infected with *S. haematobium* (2) or *S. mansoni* (1) were given miracil D by mouth at 8 a.m. each day for 6 days. Blood was withdrawn at appropriate intervals and the concentration of miracil was determined according to the method of Latner, Coxon, and King (1947) as used in the work of the previous paper. These patients, who were confined to bed, suffered no toxic effects. They left hospital six days after treatment, at which time they were still passing viable eggs. The details of these estimations are shown in Table VI, which should be compared with Table II and Fig. 2 of the paper by Hawking and Ross (1948). The blood concentrations reached in different individuals vary somewhat; allowing for these variations the concentrations obtained in these African patients were similar to those found in the European volunteers. There was no definite tendency of the compound to accumulate in the blood.

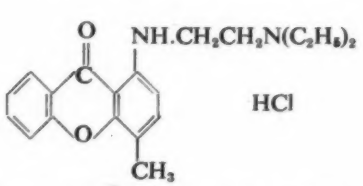
TABLE VI

CONCENTRATIONS OF MIRACIL D IN BLOOD IN MG. PER LITRE

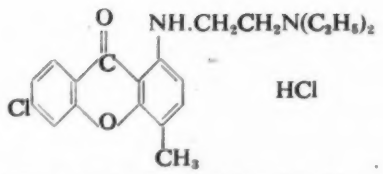
Daily dose mg.	1st day			2nd day		4th day		6th day	
	2½ hr.	6 hr.	24 hr.	2½ hr	24 hr.	2½ hr.	24 hr.	2½ hr.	24 hr.
100	—	0.62	0.42	0.53	0.24	0.32	0.32	0.52	0.28
200	0.33	0.77	0.68	0.82	0.32	0.37	0.52	0.60	0.44
300	0.89	1.20	0.96	1.20	0.90	1.10	0.78	1.40	1.20

Patients treated with miracil A, B, and C

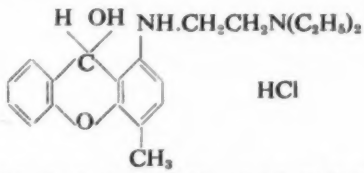
Trials were also carried out on miracils A, B, and C, but as only a small quantity of each compound (less than 10 g.) was available, which had been kindly supplied by Dr. Mauss for another purpose, only one patient could be treated with each.



Miracil A is the hydrochloride of 4-β-diethylaminoethylamino-1-methylxanthone



Miracil B is the hydrochloride of 8-chloro-4-β-diethylaminoethylamino-1-methylxanthone



Miracil C is the hydrochloride of 4-β-diethylaminoethylamino-1-methylxanthydrol

Miracils A and B are xanthones whereas miracil D is a thioxanthone. Miracil C is the dihydro derivative of miracil A ; miracil B differs from miracil A in containing a chloro substituent in the second benzene ring.

These compounds were synthesized by Mauss, and their chemotherapeutic activity on experimental schistosomiasis in mice and monkeys was investigated by Kikuth and Gönnert. They found that these compounds are well tolerated by mouth ; when given subcutaneously they produce marked local irritation which occasionally leads to necrosis at the site of the injection ; when given intravenously they are inactive except miracil D, which is not given by this route for pharmacological reasons. When given orally for the treatment of *mouse schistosomiasis* miracil B is almost four times as active as miracil C and D and more than eight times as active as miracil A. In the treatment of *monkey schistosomiasis* the relative activities are different. Miracil D is the best preparation, miracil B produces an effect only in doses which cause vomiting, and miracil A, which is only slightly active in mice, shows clear-cut activity in monkeys. Miracil C occupies a mid-way position in activity in both mouse and monkey tests.

TABLE VII

TOXICITY TESTS

The toxicity of the miracil compounds, when given orally to mice

Compound	Maximum tolerated dose in mg. per 20 g.	
	Kikuth and Gönnert (single dose)	Sewell (four daily doses)
Miracil A	6.7	5
Miracil B	20	10
Miracil C	10	5
Miracil D	13	5–10

The toxicity of these compounds was kindly determined for us at the National Institute for Medical Research, London, by Mr. P. Sewell, by giving oral doses of 5, 10, and 20 mg./20 g. mouse daily for four successive days. His results are given in Table VII and compared with those of Kikuth and Gönnert, obtained by treating mice with a single oral dose. In view of the small number of mice used, the results obtained are in agreement.

Clinical trial

The patients were young Africans. Each patient is considered individually.

A. Weight 63 kg. ; infected with *S. haematobium* ; complained of blood in urine before commencing treatment ; previously treated with antimony for schistosomiasis in January, 1947. He was treated with 6 daily doses of 100 mg. miracil A by mouth in first week and 6 daily doses of 200 mg. miracil A in second week. Total dose = 1.8 g.

B. Weight 66 kg. ; infected with *S. haematobium* ; this man had no symptoms at commencement of treatment. He was treated with 5 daily doses of 100 mg. miracil B by mouth in first week and 5 daily doses of 200 mg. miracil B in second week. Total dose = 1.5 g.

C. Weight 66 kg. ; infected with *S. haematobium* ; this man, who was very dull mentally, complained of vague "falling turns" for past three years. He was treated with 6 daily doses of 100 mg. miracil C by mouth in first week and 6 daily doses of 200 mg. miracil C in second week. Total dose = 1.8 g.

These three patients had viable eggs in their urines when treatment began. They were examined at weekly intervals during treatment, and at 2 weeks, 4 weeks, and 8 weeks after the end of treatment. On each occasion viable eggs were found in the urine. Neither these three patients nor the patients who were treated in determining the blood concentrations showed any toxic effects of the drugs.

Concentrations of miracils A, B, and C in the blood of patients

Adult African patients who were in hospital suffering from some other condition as well as schistosomiasis were used in determining the blood concentrations of these drugs. The concentration of the drug was measured by means of a Spekker photo-electric absorptiometer according to the

TABLE VIII

BLOOD CONCENTRATIONS OF THE MIRACIL COMPOUNDS AFTER A SINGLE ORAL DOSE OF 200 MG.

Compound	Mg. per litre at various times after dose		
	2½ hr.	6 hr.	24 hr.
Miracil A	0.16	0.12	Nil
Miracil B	0.10	0.28	Nil
Miracil C	Nil	Nil	Nil

method of Latner, Coxon, and King (1947) for the estimation of miracil D. It appeared that miracils A, B, and C could be estimated in the same way as miracil D. For each drug the blood concentrations after a single dose of 200 mg. were determined and these are shown in Table VIII. The blood concentrations of miracil A and miracil B were less than those observed after a single dose of miracil D (Hawking and Ross, 1948; Table II) if allowance is made for the marked individual variations between different patients. No drug could be detected in the blood of the patient who had received miracil C; as there was no more miracil C available, this result could not be verified. The blood concentrations of miracils A and B were followed in patients who received daily doses of 100 and 200 mg. for 5 days as shown in Table IX; the urinary concentration of the drugs was determined on the two days following cessation of treatment. The results are shown in Table IX. The blood concentrations of miracil A and B are lower than those observed in patients treated with miracil D in similar dosage (Hawking and Ross, 1948).

TABLE IX

BLOOD AND URINE CONCENTRATIONS OF MIRACILS A AND B DURING FIVE DAILY DOSES BY MOUTH

Daily dose of compound	Blood concentrations. mg./l.				Urine concentrations. mg./l.	
	1st day		2nd day 6 hr.	5th day 6 hr.	6th day	7th day
	2½ hr.	6 hr.				
Miracil A 100 mg.	0.20	0.10	0.40	0.32	2.0	Nil
Miracil A 200 mg.	0.30	0.46	0.56	0.48	2.7	Nil
Miracil B 100 mg.	0.16	0.04	0.08	0.28	1.2	Nil
Miracil B 200 mg.	0.24	0.10	0.30	0.34	2.1	Trace

Owing to the limited supplies of the compounds which were available the patients observed were too few to yield significant results, but they may provide preliminary indications for further investigations. In these doses no toxic effects and no therapeutic effects were observed. Judging by analogy with miracil D, however, it is probable that much larger doses would have been tolerated and these might have been therapeutically effective.

Discussion

This investigation on the therapeutic value of miracil D is still in progress and it will probably be several years before a reliable evaluation of the compound can be made. The present paper reports the preliminary results which have been obtained for the information and guidance of workers who may be interested in this subject in other parts of the world.

The therapeutic effect of miracil D seems to depend much more on the intensity of the dosage than on its total amount. At 0.2 or 0.3 g. per person (4 mg. per kg.) daily, the therapeutic effect is slight and dubious even though treatment be continued for over three weeks. At 0.5–0.7 g. per person (over 10 mg. per kg.) daily the therapeutic effect is marked, and a high proportion of the patients can be sterilized.

These results may be compared with those reported from Egypt in three papers by Azim, Halawani, and Watson (1948), Watson, Azim, and Halawani (1948), and Halawani, Watson, Nor El-Din, Hafez, and Dawood (1948). They treated patients with doses up to 7.5 g. during 8 days, but permanent cures were not usually produced except by the highest doses; toxic symptoms seem to have been more pronounced than they were in Rhodesia.

The data about the possible toxicity of the compound are conflicting. One boy (second series) suffered from generalized pruritus and a herpetiform rash after taking 6.6 g. during 15 days; this may have been due to the drug or to disintegration products of dying schistosomes. One man suffered from intense nausea, with vomiting, profound malaise, cough, and slight disorientation after taking about 14 daily doses totalling about 2.5 g.; these symptoms may or may not have been due to the drug. Some of the boys in the second series complained of nausea and colic, but many of these symptoms may have been psychological. Of the 11 patients given the biggest doses, seven were all right and five suffered from nausea, vomiting, and abdominal pain; but in only one patient

were the symptoms severe enough to require reduction of the dose.

Taking the patients as a whole, it may be concluded that nausea, vomiting, and abdominal pain may occur ; but they are not serious and their frequency is not proportional to the dose. It is not certain that the other untoward effects are really due to the drug. All these symptoms of toxicity seem to be idiosyncrasies depending on the patient more than on the size of the dose. No uniform set of toxic symptoms has yet manifested itself. The yellow staining which is seen with light skins is unimportant except from the cosmetic point of view. The insomnia, headache, giddiness, vertigo, excessive sweating, tremor, and twitching observed by Halawani and his colleagues (1948) have not been seen in our patients, nor has there been any evidence of disturbance of the heart. Halawani, Newsome, and Wooton (1947) have shown that the blood concentration of miracil (and presumably also its toxicity and its therapeutic potency) is raised by impairment of kidney function ; most of our patients were vigorous adolescents in whom the kidney function was presumably good.

The ultimate value of a drug depends on the ratio between its therapeutic effectiveness and its toxic effects, but convenience of administration, expense, and the possibilities of alternative treatments have to be taken into consideration. With miracil D evidence has now been obtained that a large proportion of patients infected with *S. haematobium* can be sterilized by doses which are tolerated by the great majority of persons. (The expense of manufacture may, however, be a handicap to widespread use.)

The effect on *S. mansoni* is not clearly indicated by the cases treated to date in S. Rhodesia. In the first series of patients, 11 out of 40 cases of *haematobium* infections were cured, but only 1 out of 11 cases of *mansoni* infections. In the second series, patient 3 had a double infection, neither of which responded. In the third series, patient 28 had a double infection ; *haematobium* ova disappeared temporarily from the excreta, but *mansoni* ova were found at all examinations (figures incomplete). There is a suggestion that *S. mansoni* responds less readily than *S. haematobium*.

Further judgment about the value of miracil must be withheld until greater experience has defined more accurately the maximum tolerated dose, has shown whether dangerous idiosyncrasies exist, and has enabled the therapeutic effect of large doses to be compared accurately with that of antimonials. In any case, miracil is of great scientific importance as showing antischistosome action in a new series of organic compounds, and it has the practical advantage of being effective when given by mouth. A safe drug which could be given orally for the treatment of schistosomiasis would be of very great value in Africa. It could be given in villages to out-patients, and administration could be left to non-skilled hands. If the effectiveness and safety of miracil are confirmed by wider experience, village treatment of schistosomiasis free from the thraldom of an intravenous ritual will at last become a practical possibility.

Summary

1. Miracil D (1-methyl-4-β-diethylaminoethylaminothioxanthone hydrochloride) is a new synthetic remedy for schistosomiasis. It is given by mouth.

2. Forty young persons infected with *S. haematobium* were treated with total doses of approximately 80–100 mg. per kg. during periods of approximately three weeks, mostly as daily doses of 0.2 g. per person (4 mg. per kg.). Twelve weeks later eleven of them were free from infection. Eleven infections with *S. mansoni* (mostly the same patients as those with *S. haematobium*) were similarly treated ; one of them became free from infection.

3. Thirteen boys infected with *S. haematobium* were treated with doses ranging from 35 to 150 mg. per kg. during fifteen days, given (irregularly) as daily doses of 0.6 g. per person. Three weeks after treatment ten out of thirteen no longer passed living ova. Four months later four of the boys appeared permanently cured.

4. Eighteen school-children infected with *S. haematobium* were given total doses of about 90–100 mg. per kg. during ten days, mostly as daily doses of 0.5–0.7 g. per person (about 10 mg. per kg.). Six weeks later fourteen out of sixteen no longer passed living ova ; fifteen weeks later twelve out of eighteen appeared to be permanently cured. Fifteen other patients (many adults) were given amounts up to 1.6 g. daily.

5. In most patients the compound was well tolerated. Symptoms of toxicity consisted mostly of abdominal pain, nausea, and vomiting. They were not directly related to the size of the dose and seemed to depend at least partially on idiosyncrasy of the individual patient.

6. Miracil D appears to exert a valuable therapeutic effect on infections of *S. haematobium* in doses which are well tolerated by most patients.

The dose should be at least 10 mg. per kg. per day. The effect of maximum tolerated doses on infections of *S. mansoni* is not yet known.

7. Three single patients were treated with miracil A, B, and C respectively in total doses of 1.5–1.8 g. during two weeks. No toxic or therapeutic effects were observed at this dosage, which is probably much less than the tolerated one.

8. In patients taking about 3 mg. miracil D per kg. daily, the blood concentration at 2½ hours after the dose was 0.3 to 0.8 mg. per 100 ml. In patients taking miracil A and miracil B, the blood concentrations were similar to (or lower than) those taking miracil D. In a patient given miracil C, the compound could not be detected in the blood.

Grateful acknowledgments are due to Dr. R. M. Morris, medical director, Southern Rhodesia, for access to clinical material and laboratory facilities; to the principals of the schools involved for their kind co-operation; and to Drs. Mauss, W. Kikuth, and R. Gönnert for information and material.

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ANTIMALARIAL COMPOUNDS AS ANTAGONISTS OF ADENOSINE

BY

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The possible antagonism of the action of adenosine on some tissues by antimalarial drugs has been studied with the object of obtaining some information on the mode of action of this type of drug.

METHODS

Antagonism of adenosine

The following techniques were used in these experiments:—

(1) The isolated hen's caecum.—The action of adenosine on this tissue (Barsoum and Gaddum, 1935) was studied before and after the addition to the bath of known concentrations of antimalarials. Although it was possible to demonstrate an antagonism between these compounds and adenosine by this method, satisfactory results were not obtained because of the intense relaxation of the caecum produced by the antimalarial substances themselves.

(2) The measurement of the duration of the auriculoventricular (a.v.) block produced by intravenous administration of adenosine in the anaesthetized guinea-pig (Drury and Szent-Györgyi, 1929). The guinea-pig's heart is not so sensitive to adenosine as the hen's caecum, but this method has the advantage that the antimalarials do not alter the electrocardiogram (e.c.g.) in sub-toxic doses.

Three series of experiments were carried out with this technique.

(*a*) In the first series, hereafter called "acute," the compounds under test were administered by slow intravenous injection. Guinea-pigs of 250–400 g. bodyweight were anaesthetized with pentobarbitone (nembutal), given intraperitoneally. A cannula was inserted into one of the jugular veins and the animals maintained under artificial respiration during the experiments by means of a Palmer's Starling Universal pump for small animals; $1:10^{-4}$ or $1:10^{-3}$ adenosine in normal saline was administered intravenously by the jugular vein cannula, never more than 1 c.c. of adenosine solution being injected. Electrocardiograms were taken, lead II, immediately after the administration of adenosine by means of a Cossor electrocardiograph modified for fast heart rates.

In each experiment a series of doses of adenosine from 0.1 to 0.3 mg./kg. was first administered in order to produce varying degrees of a.v. block. The cannula was then washed with the solution of antimalarial and the drug slowly infused by means of a motor-driven syringe which delivered the solution at the rate of 0.02 ml./min. This infusion was maintained for 30 min., after which the cannula was washed with adenosine solution and a second series of adenosine injections was administered in doses large enough to produce measurable heart blocks.

The dose of antimalarial was calculated in mg./kg./min. and adjusted by varying the concentration according to the body-weight of each animal. At least 3 guinea-pigs were used for each dose level of antimalarial.

This series was used mainly for the selection of chemical types able to antagonize the action of adenosine.

(*b*) In the second series the compounds were administered orally in a dosage regime similar to that used by Davey (1946) for the assessment of antimalarial activity against *P. gallinaceum* in chicks.

Batches, usually of 6 guinea-pigs each, were used for each dose level of every compound tested. The animals received two doses daily for 3½ days, a total of 7 doses. The drugs were administered in solution in water, or in fine suspensions when insoluble, by means of a stomach tube. After the 7th dose, the guinea-pigs were anaesthetized with pentobarbitone and a series of adenosine injections was administered as described above.

These experiments will be referred to as "chronic" later on.

(*c*) Some experiments were carried out with heart-lung preparations of guinea-pigs. The method was essentially that described by Cruickshank (1945) for the rat; 25 ml. of heparinized guinea-pig's blood were used in the apparatus. The elastic peripheral resistance was adjusted to give a pressure of 80–100 mm. Hg in the aortic cannula; e.c.g. records were taken with leads placed in the superior vena cava and in the apex of the heart. Adenosine was injected into the venous cannula of the preparation.

Assessment of the adenosine action and of the antagonistic action of antimalarials

The action of adenosine in normal and treated guinea-pigs was assessed by the duration of the a.v. block as recorded in the electrocardiograms obtained during experiments. The duration of this action was measured as the time which elapsed between the first and last P wave not followed by a ventricular complex. No attempt was made to measure the duration of these effects by the inspection of the duration of the P–R intervals. From these results, the doses of adenosine which would have produced a.v. blocks of 5 seconds' duration were calculated by graphical methods.

An attempt to compare the intensity of the adenosine antagonism shown by the substances under test was made by calculating the doses of antagonists which would reduce the sensitivity of the guinea-pigs to adenosine to one-half of the normal (see Tables II and III).

The results so obtained were only semi-quantitative and can only be taken as a rough measurement of the intensity of the antagonism.

Antimalarial activity

The data on the antimalarial activity of the drugs used in this work was obtained in our laboratories by Dr. D. G. Davey using chicks infected with *Plasmodium gallinaceum* (Davey, 1945). Most of these results have already been published in the papers dealing with the synthesis of the compounds in question.

Materials

The substances used in this work can be classified in the groups indicated in Table I. In this Table the chemical constitution and the trivial name or code number of each compound is given. In the text these numbers and names will be used in preference to the full chemical names. In each group substances have been selected with as low and as high antimalarial action as possible.

Most of the substances used in our work were prepared in these laboratories (Curd and Rose, 1946a; Curd, Davies, and Rose, 1946; Curd, Davies, Owen, Rose, and Tuey, 1946; Curd, Richardson, and Rose, 1946; Curd and Rose, 1946b; Basford, Curd, and Rose, 1946; Curd and Rose, 1946c; Curd and Landquist, 1948; Curd, Landquist, and Rose, 1947). Others were obtained from Professor A. R. Todd (Hull, Lovell, Openshaw, Payman, and Todd, 1946; Hull, Lovell, Openshaw, and Todd, 1947). Quinidine and hydroquinidine were a gift of Messrs. Amsterdamshe Chininefabriek, Amsterdam. The adenosine used was purchased from Messrs. British Drug Houses, Ltd.

Results

(1) Antagonism in the hen's caecum

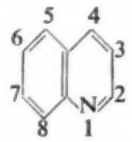
This antagonism was studied by measuring the relaxation of this tissue produced by known concentrations of adenosine in normal Tyrode's solution and in Tyrode's solution containing known quantities of antimalarial.

Mepacrine usually had practically no visible effect on the caecum in concentrations of $1 : 10^{-7}$ to $1 : 10^{-6}$, but it decreased the action of adenosine in this concentration range. Higher concentrations caused an intense relaxation of the caecum and at the same time reduced still further the action of

TABLE I

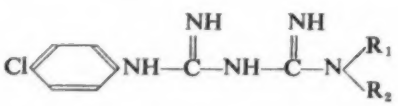
CHEMICAL COMPOSITION OF SOME OF THE ANTIMALARIAL AND RELATED COMPOUNDS USED

Quinolines



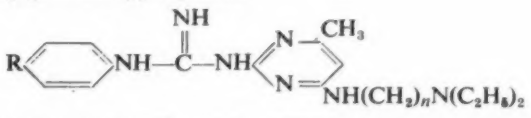
Name or code number		Substituents
Pamaquin ..	6 8	OCH_3 $NH.CH(CH_3)(CH_2)_3N(C_2H_5)_2$
Chloroquine	4 7	$NH.CH(CH_3)(CH_2)_3N(C_2H_5)_2$ Cl
5735 ..	2 and 3 4 7	CH_3 $NH.CH(CH_3)(CH_2)_3N(C_2H_5)_2$ Cl
3738 ..	2 4 7	OH $NH(CH_2)_3N(C_2H_5)_2$ Cl

Biguanides



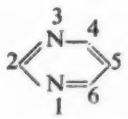
Name or code number	Paludrine	4430	5093
R_1	$CH(CH_3)_2$	$CH(CH_3)_2$	CH_3
R_2	H	CH_3	H

Arylguanidinopyrimidines



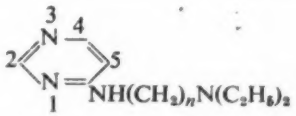
Code number	3349	3749	3742
R	Cl	Cl	CH_3O
n	2	3	2

Anilinopyrimidines



Code number	Substituents			
	2	4	5	6
2666	(*p*)Cl.C_6H_4NH	CH_3		$NH(CH_2)_2N(C_2H_5)_2$
3300	(*p*)Cl.C_6H_4NH	CH_3		$NH.CH(CH_3)(CH_2)_3N(C_2H_5)_2$
4070	CH_3	(*p*)Cl.C_6H_4NH		$NH(CH_2)_2N(C_2H_5)_2$
3756	(*p*)Cl.C_6H_4NH	—CH = CH — CH = CH—		$NH(CH_2)_2N(C_2H_5)_2$
4316	$(C_4H_9)_2N(CH_2)_3NH$	CH_3		(*p*)Cl.C_6H_4NH

Pyrimidines



Code number	Substituents			
	2	4	5	*n*
4747	CH_3	CH_3		2
4419	CH_3	NH_2	CH_3	2
3920	NH_2	CH_3		2
3448	NH_2	CH_3	CH_3	2
4746	NH_2	NH_2		3
4420	CH_3	NH_2		3
4450	CH_3	CH_3	$CH_2C_6H_5$	3
4184	NH_2	$—CH_2CH_2CH_2CH_2—$		3

adenosine. Fig. 1 summarizes the results of one of these experiments.

Similar results were obtained with quinine, 3349, and 2666, but the intense relaxation of the caecum produced by each of these antimalarials made the assessment of the antagonism somewhat difficult. For this reason this method was abandoned after a few trials with different antimalarial drugs.

(2) *Antagonism between adenosine and antimalarials on guinea-pig's heart*

The duration of the a.v. block produced by the intravenous administration of adenosine is proportional to the dose. The minimum effective dose of adenosine, in guinea-pigs anaesthetized with pentobarbitone, was 0.1 mg./kg., and this generally produced a block of short duration during which one or two independent P waves were recorded. The average duration of this block in a series of 40 guinea-pigs was found to be 0.67 sec. Blocks of 10–15 sec. duration were generally obtained with 0.3 mg./kg. of adenosine. The mean durations of the blocks produced in this control series with doses of adenosine from 0.1 to 0.3 mg./kg are given in Table III.

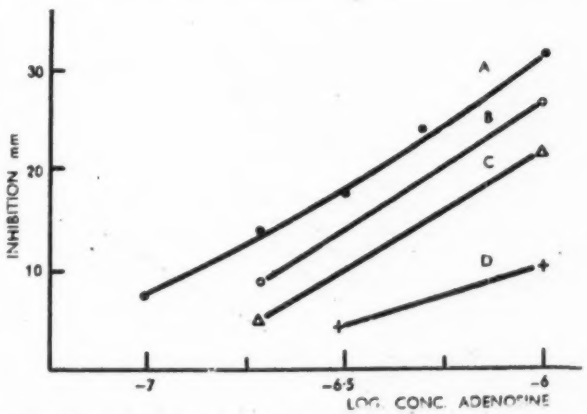


FIG. 1.—Influence of mepacrine HCl on the response of the hen's caecum to adenosine. Abscissae—log concentration of adenosine. Ordinates—relaxation of caecum in millimetres. A—normal Ringer. B—in mepacrine 10^{-7}. C—in mepacrine 10^{-6}. D—in mepacrine $10^{-5.5}$.

TABLE II

MINIMAL DOSES WHICH ANTAGONIZE THE ACTION OF ADENOSINE IN ACUTE EXPERIMENTS

Drug	Minimum antagonistic dose mg./kg./min.	Dose which halves sensitivity to adenosine mg./kg./min.
Quinine HCl ..	0.75	0.8
Mepacrine HCl	0.25	0.35
Pamaquin HCl	too toxic at 0.25	..
Chloroquine H_3PO_4 ..	0.25	0.45
Paludrine lactate	1.25	1.25
3349	3.0(?)	3.0(?)
2666	0.6	0.9
Procaine ..	Inactive at 2.0	..
Suramin ..	,, at 5.0	..
Methylene blue	,, at 2.0	..
Trypan blue ..	,, at 2.0	..
Trypan red ..	,, at 2.0	..
Sulphadimethyl-pyrimidine ..	,, at 8.5	..

A. "Acute" experiments

Table II gives the minimum antagonistic doses of the compounds tested in this series and the doses which reduce the action of adenosine to one-half.

Mepacrine

The duration of the blocks produced by adenosine after the continuous administration of 0.1 mg./kg./min. mepacrine for 30 min. did not differ significantly from that obtained before the antimalarial had been given. An appreciable reduction of the duration of the action of adenosine was observed after the administration of 0.25 mg./kg./min. mepacrine. The decrease in the effects of adenosine was more marked after 0.35 and 0.5 mg./kg./min. of antimalarial had been given. Fig. 2 shows these results.

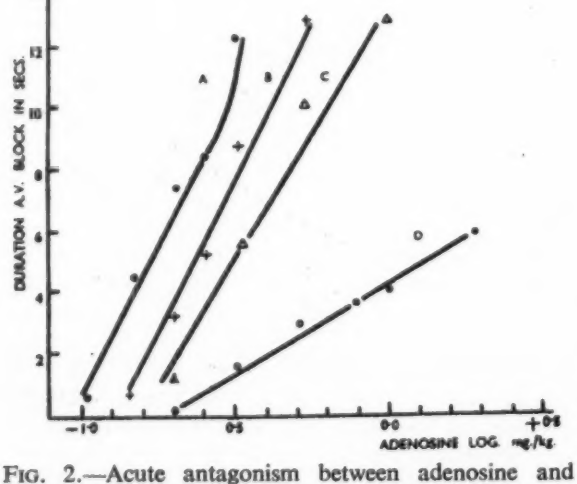


FIG. 2.—Acute antagonism between adenosine and mepacrine in the guinea-pig. Duration of the a.v. block produced by adenosine in guinea-pig after a continuous injection of mepacrine HCl for 30 min. Abscissae—log. dose of adenosine in mg./kg. Ordinates-duration of block in seconds. A—before administration of mepacrine. B—after 0.25 mg./kg./min. mepacrine. C—after 0.35 mg./kg./min. mepacrine. D—after 0.5 mg./kg./min. mepacrine.

This antagonism, however, is reversible. When the response to adenosine was examined at different times after the end of the infusion of mepacrine, it was found that the duration of the block became progressively longer, and in 90 min. the adenosine effects were about the same as those of the control series (Fig. 3).

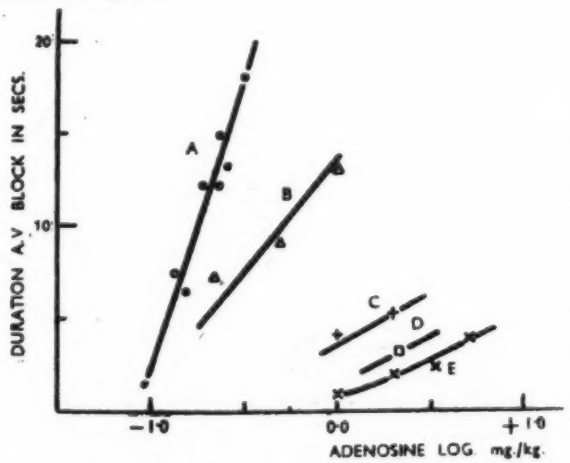


FIG. 3.—Duration of the a.v. block produced by adenosine before and at different times after the end of the continuous injection of mepacrine HCl of 0.5 mg./kg./min. for 30 min. Abscissae-dose of adenosine as log mg./kg. Ordinates-duration of block in seconds. A (dots)—before mepacrine. E—immediately after mepacrine. D—15 min. after mepacrine. C—30 min. after mepacrine. B—60 min. after mepacrine. A (circles)—90 min. after mepacrine.

Pamaquin

It was impossible to study the action of pamaquin by this method because of its high toxicity. Doses as small as 0.25 mg./kg./min. of pamaquin HCl were too toxic and caused bradycardia and right predominance in the e.c.g. Larger doses of pamaquin HCl killed the guinea-pigs before the end of the infusion period.

Chloroquine

Chloroquine is less toxic than pamaquin. Doses from 0.25 to 1 mg./kg./min. were administered without causing undue alterations of the e.c.g. The

minimum doses of chloroquine that antagonize the action of adenosine are of the order of 0.25 mg./kg./min.

Quinine

Quinine was inactive when administered in doses of 0.5 mg./kg./min. A good reduction in the duration of the a.v. block produced by adenosine was observed after doses of 0.75 mg./kg./min. had been infused.

Paludrine

Paludrine was found to be active in doses from 1.0 mg./kg./min. and the adenosine antagonism was very marked with 2 mg./kg./min. In some of these experiments the paludrine concentration of the blood was as high as 7 to 9 mg./l.

3349

3349 was found to be even less active than paludrine in antagonizing the effects of adenosine in this "acute" series. Doses of 2 mg./kg./min. were not antagonistic; doses of 3 mg./kg./min. showed a clear antagonism but were too toxic to the heart. The e.c.g. tracing showed cardiac irregularities and bradycardia, which made it impossible to assess the intensity of the antagonism.

2666

The antagonism to adenosine with this compound was more intense than with paludrine or 3349 but less than with quinine, mepacrine, or chloroquine. Doses of 1 mg./kg./min. of 2666 were strongly antagonistic, whereas 0.5 mg./kg. had no action on the adenosine effects.

Substances without adenosine antagonism

A selection of substances, some of them without antimalarial activity, were tested by this method. Procaine, suramin, methylene blue, trypan red, trypan blue, and sulphadimethylpyrimidine were tested; none of these antagonized the adenosine effects.

B. "Chronic" experiments

The adenosine antagonism shown by antimalarials of different types was studied in more detail in this series of experiments. Details of these experiments are given in Table III, which also gives the doses of antagonists which halve the sensitivity of the animals to adenosine.

Acridine derivatives: mepacrine

The heart blocks produced by adenosine in guinea-pigs that had received seven doses of mepacrine in 3½ days were shorter than those obtained in normal guinea-pigs.

The minimum dose of mepacrine which showed this effect was 2 × 5 mg./kg. daily. Larger reductions in the duration of the adenosine effects were observed when the dose of mepacrine was increased from 5 to 40 mg./kg. twice daily. With the last-mentioned dose, the minimum effective dose of adenosine was 0.3 mg./kg., which produced the same effect as 0.1 mg./kg. in normal guinea-pigs. Details of the results of these experiments are shown in Table III and Fig. 4.

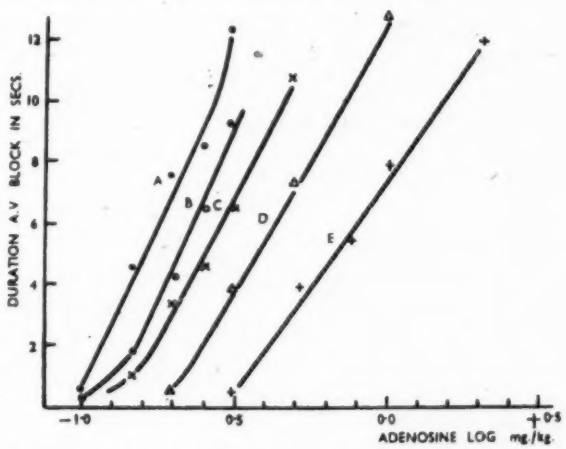


FIG. 4.—"Chronic" antagonism between adenosine and mepacrine HCl in guinea-pigs. Duration of the a.v. block produced by adenosine in guinea-pigs treated by mouth with different doses of mepacrine. Abscissae-dose adenosine as log. mg./kg. Ordinates-duration of block in seconds. A—controls. B—mepacrine HCl, 2 × 5 mg./kg./day. C—mepacrine, 2 × 10 mg./kg./day. D—mepacrine, 2 × 20 mg./kg./day. E—mepacrine, 2 × 50 mg./kg./day.

Quinoline derivatives

Pamaquin given by mouth as the methylene bis-β-hydroxynaphthoic acid salt was tolerated by guinea-pigs in doses up to 20 mg./kg. twice a day for 3½ days. A reduction of the duration of the adenosine block was obtained in guinea-pigs after treatment for 3½ days with 2 × 10 mg./kg. pamaquin salt, equivalent to 6.25 mg./kg. base.

Chloroquine diphosphate was antagonistic to adenosine in doses of from 2 × 5 mg./kg./day. The low toxicity of this substance allowed the investigation of its antagonism in a range of doses from 5 to 50 mg./kg. twice daily. Doses of adenosine that produced blocks of 5 sec. duration in guinea-pigs treated with pamaquin or chloroquine did not show any significant difference between the intensities of the antagonism produced by these two substances.

TABLE III

ANTAGONISM OF ADENOSINE BY ANTIMALARIALS ADMINISTERED ORALLY TWICE DAILY FOR THREE AND A HALF DAYS (7 DOSES)

Compound	Dose (mg./kg.)	Number of animals	Adenosine injected mg./kg. Mean duration of auriculo-ventricular block (seconds) 0.10	0.15	0.2	0.25	0.30	0.50	1.00	Dose which halves sensitivity to adenosine mg./kg.	Minimal dose for full antimalarial activity mg./kg.
Controls (no drug)		40	0.67	4.58	8.12	9.65	12.58	—	—		
Quinine (dihydrochloride) ..	50	3	0.4	1.2	2.48	3.6	5.3	—	—	60	40
	100	6	0.0	0.4	1.6	3.6	4.9	10.15	16.06		
	150	6	0.0	0.9	2.1	3.2	4.4	7.0	11.7		
	200	6	0.0	0.0	0.5	1.0	1.38	2.86	9.4		
Quinidine (base)	50	5	0.0	3.9	5.3	7.5	10.9	—	—	60	40
	100	6	0.0	0.7	1.53	2.57	3.3	—	—		
Hydroquinidine (base)	100	6	0.5	2.1	3.5	6.0	7.5	—	—	100	80
Mepacrine dihydrochloride ..	5	6	0.31	3.01	4.36	6.55	9.23	—	—	14	40
	10	5	0.0	0.45	3.21	—	6.54	15.5	—		
	20	6	—	0.12	1.75	—	3.72	7.15	12.7		
	40	6	—	—	—	—	0.65	3.46	8.2		
Pamaquin methylene bis-β-hydroxy-naphthoate	10	8	0.9	3.4	5.3	7.7	9.5	—	—	9	4
	20	12	0.25	1.86	3.7	4.1	6.88	—	—		
Chloroquine diphosphate ..	5	6	0.0	2.1	3.75	5.9	8.5	—	—	9	5
	10	6	1.5	2.3	3.55	5.05	7.4	11.4	—		
	20	5	0.0	0.0	0.6	2.6	4.0	7.8	13.2		
	50	6	—	—	0.0	0.0	0.0	4.6	7.0		
5735 dihydrochloride	20	6	0.43	1.2	1.7	2.6	4.5	—	—	33	80
	50	6	—	—	0.6	3.4	3.7	6.4	—		
3738 base	50	6	0.0	2.8	5.2	8.05	10.8	—	—	70	Inactive at 400
Paludrine lactate	10	6	0.7	—	6.8	—	11.7	—	—	15	5
	20	6	0.0	2.5	5.4	8.3	9.2	—	—		
4430 acetate	20	6	0.0	1.2	5.1	7.25	12.8	—	—	40	20
	50	6	0.0	1.7	3.9	5.9	7.5	—	—		
5093 hydrochloride	20	6	2.38	4.2	7.34	9.32	12.25	—	—	No action	Inactive at 160
	50	6	1.8	4.2	6.7	9.8	15.9	—	—		

TABLE III (*continued*)

ANTAGONISM OF ADENOSINE BY ANTIMALARIALS ADMINISTERED ORALLY TWICE DAILY FOR THREE AND A HALF DAYS (7 DOSES)

Compound	Dose (mg./kg.)	Number of animals	Adenosine injected mg./kg. Mean duration of auriculo-ventricular block (seconds) 0.10	0.15	0.2	0.25	0.30	0.50	1.00	Dose which halves sensitivity to adenosine mg./kg.	Minimal dose for full antimalarial activity mg./kg.
3349 base	20	5	0.1	2.0	3.94	5.16	6.56	—	—	20	40
	50	5	0.0	0.16	0.8	2.04	3.58	6.5	—		
	75	4	—	—	0.0	0.0	0.28	1.5	8.5		
	100	6	—	—	—	—	0.0	0.0	0.0		
3672 dihydrochloride	20	6	0.8	2.9	4.6	6.8	10.2	—	—	30	200
	100	6	—	—	0.0	—	1.0	4.2	13.2		
3742 base	20	6	0.12	5.5	8.9	10.8	14.4	—	—	Inactive	Inactive at 200
	50	6	0.0	2.48	4.7	7.2	10.1	—	—		
2666 dihydrochloride	50	6	1.5	4.5	6.6	9.7	13.2	—	—	90	80
	100	6	0.0	1.9	1.1	3.65	7.62	13.9	—		
	200	6	—	0.0	0.0	3.0	4.5	6	—		
3300 dihydrochloride	50	6	0.35	4.16	5.66	7.28	7.4	—	—	75	200
	75	6	—	—	0.8	2.55	6.0	6.56	—		
	100	5	—	—	0.66	—	1.7	3.9	6.74		
4070 base	20	6	0.86	5.4	8.2	10.5	14.5	—	—	60	Inactive at 120
	50	6	—	—	2.2	5.7	7.0	13.4	—		
	100	6	—	—	1.1	—	4.3	8.1	17.72		
3756 base	20	6	0.5	6.26	9.3	10.4	15.2	—	—	No action	Slightly active at 40
	50	6	0.0	2.5	5.5	6.9	9.2	—	—		
4316 dihydrochloride	100	6	0.0	2.1	3.6	6.0	8.2	—	—	100	40
4747 base	50	6	—	4.1	7.2	10.5	11.05	—	—		Trace activity at 240
4419 base	50	6	—	1.8	2.7	4.4	6.2	—	—		80
3448 base	50	6	—	—	0.4	—	2.8	6.1	10.7		100
4746 base	50	6	0.6	3.9	4.5	7.2	9.5	—	—		Trace activity at 400
4420 base	50	6	0.0	0.4	1.2	2.6	5.5	—	—		80
4450 base	50	4	—	—	—	0.27	0.35	4.9	4.0		80
4184 base	50	4	—	—	0.3	1.02	3.1	6.9	10.9		120

The antagonism to adenosine shown by some derivatives of chloroquine such as 5735 and 3738 was weaker than that of the parent compound. The order of intensity of the antagonism shown by these substances was practically parallel to that of their antimalarial activity. Chloroquine and pamaquin, both of which have high antimalarial activity, antagonize adenosine better than 5735, which has low antimalarial activity. 3738, which has hardly any antimalarial action, did not modify the action of adenosine.

Cinchona alkaloids

The cinchona alkaloids studied in this series were quinine, quinidine, and hydroquinidine. In the experiments with quinine it was observed that the antagonism to adenosine was marked in guinea-pigs for 2 hours after the last dose of alkaloid, but in experiments carried out later than that adenosine produced blocks of about the same duration as in the controls. As it is known that quinine is metabolized in the body fairly quickly, it was thought that these differences in the results were due to the rapid disappearance of the drug from the blood. For this reason the dosage schedule for cinchona alkaloids was modified in the following way: The six guinea-pigs used for each dose level received their seventh dose of drug at the usual time, about 9.30 a.m., and three of them were used before 12 noon. The remaining three received an eighth dose at about 1.30 p.m. and were used immediately after. With this modification of the dosage regime the results for both groups were about the same.

Quinine and quinidine were adenosine antagonists in guinea-pigs when doses larger than 50 mg./kg. were administered twice daily. The intensities of the antagonism to adenosine produced by these two alkaloids were practically the same, and it is interesting to note that both are equally active against experimental malaria.

Hydroquinidine, which as an antimalarial is about half as active as quinine, produced a milder action than the latter.

N^1-phenyl-N^5-alkylbiguanides

In the series of drugs of the paludrine type we selected one drug without antimalarial activity (5093), and compared it to 4430 and paludrine itself (4888). The inactive compound was found not to antagonize the action of adenosine even in the maximum tolerated doses.

Paludrine antagonized adenosine when doses from 2 × 10 mg./kg./day were administered to guinea-pigs. Unfortunately it was not possible to increase the dose beyond 2 × 20 mg./kg./day ; all guinea-pigs treated with this dose survived this treatment for 4–5 days, but although a small increase of the dose to 2 × 30 mg./kg./day did not kill any animal during the first 1½–2 days it produced a 100 per cent mortality if the treatment was maintained for a longer time. This high toxicity of paludrine, when given repeatedly to guinea-pigs, contrasts with its moderately low toxicity when given in single doses. Similar results have been found by Butler, Davey, and Spinks (1947) in other laboratory animals, and it is probable that they are due to the formation and accumulation of some highly toxic metabolite of paludrine during the period of repeated administration.

The minimum dose of 4430 which antagonized the action of adenosine was 2 × 30 mg./kg./day. This drug has a lower antimalarial activity than paludrine and at the same time it is a weaker antagonist.

Arylguanidinopyrimidines (Curd and Rose, 1946a)

A similar gradation of antagonistic activity was found among the substances of this type, and their antagonistic activity ran parallel to their antimalarial action. 3349 has an antimalarial activity and a capacity to antagonize adenosine similar to those of paludrine. 3672 is a weaker antagonist and a weaker antimalarial, and finally 3742, which has no action on malarial parasites, does not antagonize adenosine.

Anilinopyrimidines (Curd and Rose, 1946b ; Curd, Davies, and Rose, 1946 ; Curd, Davies, Owen, Rose, and Tuey, 1946 ; Curd, Richardson, and Rose, 1946)

The prototype of this group of substances (2666) was the first substance synthesized by Curd and Rose that showed antimalarial activity in chicks. The results of its clinical trials were disappointing, and according to Adams it is not active in human malaria (quoted by Curd, Davey, and Rose, 1945).

This group of substances was the only one in which it was impossible to find any relation between antimalarial activity and adenosine antagonism. Two members of the group, 3300 and 4070, are devoid of antimalarial activity but they were, nevertheless, good antagonists of adenosine. 2666 itself is a substance with a fairly good antimalarial activity, but it inhibited the action of adenosine more intensely than 4316, which is a better antimalarial. On the other hand 3756, which is inactive against experimental malaria, did not show any appreciable antagonism to adenosine.

Alkylpyrimidines (Hull, Lovell, Openshaw, Payman, and Todd, 1946; Hull, Lovell, Openshaw, and Todd, 1947)

Because only small samples of these compounds were available, they were not tested in a full range of doses. For this reason it was neither possible to study the relative order of the intensities of their antagonisms nor to compare these with their antimalarial activities. All of them were tested at 2 × 50 mg./kg./day, which as a rule with most antimalarials produced a measurable antagonism. It is interesting to note that compounds 4747 and 4746, which are inactive against *P. gallinaceum*, did not antagonize the action of adenosine under the conditions of the test. Under the same conditions, all the other compounds tested in this series produced a clear antagonism. The details of the results of these experiments are reproduced in Table III.

Relation between antimalarial activity on chicks and adenosine antagonism

As already stated above, within all groups of substances, except the 2666 type, there is a parallelism between their antimalarial activity and their power to antagonize adenosine, as expressed by the dose which halves the sensitivity of the animals to this substance. In comparing the effects of the substances selected as prototypes for each group it was found that mepacrine was one of the best antagonists of adenosine, in spite of the fact that it is one of the weakest antimalarials in chicks. Among the other substances, however, the best antimalarials are among the best antagonists, and substances with low antimalarial activity are among those which antagonize adenosine feebly. Table IV gives the orders of antimalarial activity and intensity of the antagonism shown by these substances in the "acute" and "chronic" experiments.

TABLE IV

Adenosine antagonism		Antimalarial activity
"Acute"	"Chronic"	
Mepacrine	Pamaquin	Pamaquin
Chloroquine	Chloroquine	Chloroquine
Quinine	Mepacrine	Paludrine
2666	Paludrine	3349
Paludrine	3349	Mepacrine
3349	Quinine	Quinine
	2666	2666

C. Antagonism in the heart-lung preparation

In the experiments reported above it was found that paludrine antagonized adenosine in both "acute" and "chronic" experiments. When the intensity of its "chronic" antagonism was compared with that of other prototype substances it was found that the order of intensity of their antagonism was about the same as that of their antimalarial activity. However, in the "acute" series this parallelism was not maintained and under these conditions paludrine was found among the worst antagonists (Table IV).

Another fact noted was the delayed toxic effect of paludrine when administered repeatedly. A likely explanation of this toxic action may be the production and accumulation of some toxic metabolite during the prolonged administration of paludrine.

It is also possible that the difference in the results obtained with paludrine in the "chronic" and "acute" experiments may be due, not to the drug antagonizing adenosine by itself, but to its being converted during the dosing period into a metabolite which does antagonize adenosine. Even when the paludrine content of the blood was as high as 7–9 mg./l. in the "acute" experiments, the antagonism was not intense, but during the brief period of slow administration of the drug only a small proportion of the injected material could have been metabolized into the hypothetical active substance.

The possibility of such a metabolite being formed was investigated in the heart-lung preparation of the guinea-pig in the following way: e.c.g. records of the action of several small doses of adenosine (from 25 μg.) were taken before and after the addition of paludrine to the blood of the preparation. The blood was then replaced by the same volume of fresh normal blood, which was left to circulate for 15 min.; this "washing out" of the drug was repeated and finally the blood was replaced by blood from guinea-pigs which had been treated orally with 2 × 20 mg./kg. of paludrine for 3½ days. This last change of blood was repeated and 25 ml. of blood from paludrine-treated guinea-pigs were again put in the venous reservoir. A further series of adenosine doses was injected 15 min. after the last change of blood.

With normal blood in the preparation the injection of about 25 μg. adenosine into the venous cannula produced blocks of about 5 sec. duration. The addition of paludrine to the blood of the preparation caused no reduction in the duration of the effects of adenosine, but a significant reduction of these effects was observed when the blood

of paludrine-treated guinea-pigs circulated in the preparation.

The concentration of paludrine in the blood of the treated guinea-pigs and that in the blood to which paludrine had been added was estimated by the method of Spinks and Tottey (1946). In some experiments, such as that reproduced in Table V, the concentration in the blood of the treated animals was smaller than that in the blood to which paludrine had been added, but nevertheless the blocks produced by the same doses of adenosine were shorter when the blood of paludrine-treated animals circulated in the preparation.

TABLE V

ANTAGONISM TO ADENOSINE BY ANTIMALARIALS IN HEART-LUNG PREPARATIONS OF GUINEA-PIGS

Expt. No.	Blood (25 ml.) and drug	Duration of a.v. block in secs. with doses of adenosine, in μg.			
		25	35	50	100
2	Normal blood	1.4	—	9.4	—
	Ditto and mepacrine HCl 0.02 mg.	0.0	—	6.8	—
	Normal blood (after 3 changes)	3.4	—	11.9	—
	Ditto and mepacrine HCl 0.2 mg.	0.0	—	5.0	11.3
13	Normal blood	3.4	—	12.8	—
	Ditto and quinine HCl 0.05 mg.	3.6	—	12.6	—
	Ditto and quinine HCl 0.5 mg.	0.0	—	—	0.0
	Normal blood (after 3 changes)	0.0	—	8.4	14.0
4	Normal blood	0.0	6.2	11.3	—
	Ditto and paludrine (1.027 mg./l.)	—	6.8	10.2	—
	Blood of paludrine-treated guinea-pigs (conc. 0.82 mg./l.) (after 2 changes with blood of paludrine - treated guinea-pigs)	—	2.9	5.6	—

These results were compared with those obtained when mepacrine or quinine was added to the blood of the preparation.

The duration of the heart block produced by adenosine was reduced when mepacrine was added to the blood in concentrations of 80 μg./100 ml. This antagonism was observable so long as blood containing mepacrine circulated in the preparation. When the drug was " washed out " by two or three changes of blood the duration of the action of adenosine returned to normal values. Similar results were obtained with quinine in concentrations of the order of 2 mg./100 ml. Table V gives the results of some of these experiments.

Discussion

The antimalarial action of a number of chemical substances has been compared with their power to antagonize the responses of some mammalian tissues to adenosine. In most of the series of compounds tested a correlation has been observed between the inhibition of responses to adenosine and the antimalarial activity. This may mean that antimalarial action in some way involves interference of the drug with a process, essential to the parasite, in which adenosine or a derivative thereof is concerned.

No idea as to the nature of this hypothetical process can be gathered from the experiments reported here. It is unlikely that the processes in mono-cellular plasmodia in which adenosine or its derivatives are concerned can be closely related to the processes which we have been studying in mammalian tissues.

This study of the antagonism of the action of adenosine in some tissues by antimalarial drugs was undertaken because adenosine forms part of coenzymes, such as, for instance, flavine adenine dinucleotide. It was already known (Madinaveitia, 1946) that the antibacterial action of some antimalarial compounds on *Lactobacillus casei* was antagonized by riboflavine. It has been suggested that this effect is due to the similarity of the spatial configuration of these drugs to that of the vitamin (Curd, Davey, and Rose, 1945). This structural resemblance has also been claimed to be connected with the parasiticidal activity of these drugs (Curd and Rose, 1946a).

On the other hand the alternative hypothesis that an interference with an adenosine-containing enzyme system might result in antimalarial activity has led to the preparation of successful antimalarial compounds (Hull *et al.*, 1946, 1947). A biochemical relationship between adenosine derivatives and antimalarials may be found in the observation (Hellerman, Bovarnick, and Potter, 1946) that adenylic acid and adenosine triphosphate prevent the inhibition which mepacrine causes in the recovery of oxygen uptake by washed *Plasmodium lophurae* in the presence of glucose. The fact that some enzymes contain both adenosine and riboflavine makes both points of view on the mode of action of antimalarial substances compatible.

It is unlikely that *L. casei* uses riboflavine as such in its metabolic processes. It is more probable that the micro-organism builds up some coenzyme from the free riboflavine present in the medium, possibly by combining it through a polyphosphate group with adenosine. It is conceivable that the antagonistic effect of riboflavine in *L. casei* assays is not due to the vitamin itself, but to an adenosine-containing coenzyme into which the free riboflavine is incorporated. The prosthetic group of *d*-amino-acid oxidase is such a flavine adenine dinucleotide (Corran, Green, and Straub, 1939) and is known to antagonize the inhibitory action of mepacrine on the enzymatic deamination of *d*-amino-acids (Wright and Sabine, 1944).

On the other hand it has been shown (Haas, 1944) that mepacrine also inhibits cytochrome reductase by an irreversible reaction with the protein; here the drug has been shown to compete with the prosthetic group of the enzyme, because its action is prevented by the addition of alloxazine mononucleotide. In this coenzyme riboflavine is not combined with adenosine.

All the antimalarial drugs which we have tested have been found to antagonize the particular actions of adenosine which we have chosen to study. From the circumstantial evidence already available and that now presented it appears that some relationship exists between the ability of chemical compounds to interfere with biological processes in which adenosine is concerned and their antimalarial action.

In spite of the fact that the results of the experiments reported above were only semi-quantitative, it was found (*a*) that antimalarial drugs have another action in common—i.e., they antagonize adenosine, and (*b*) that the intensity of this antagonism is proportional to their antimalarial action as measured in chicks infected with *P gallinaceum*.

The relation of the two activities is best seen when the results obtained with a series of homologues are compared. The only exceptions to this rule were the anilinopyrimidines of the 2666 type among which no relation between antimalarial activity and adenosine antagonism could be found. It is noteworthy that although 2666 was found to be active in the chick test, the drug was tried clinically with negative results by Dr. A. R. D. Adams, of the School of Tropical Medicine of Liverpool (cited by Curd, Davey, and Rose, 1945). However, as the chick test is only an indication of possible antimalarial activity in man, and as there is an unpredictable variability in the susceptibility of various species of plasmodia to the same drug (Davey, 1946), it is more than probable that the agreements and disagreements shown in Tables III and IV would have been different if another measurement of activity had been chosen.

In comparing the plasmodicidal activity of the prototype of each group of substances with the intensity of their adenosine antagonism in the "chronic" series, it was found that mepacrine was the best antagonist, although it is not one of the more powerful antimalarials in the chick test. There is, however, a fairly close parallelism between the antagonism and the activity of the other substances.

This relationship is not so close in the "acute" experiments. The most important exception was paludrine: in the "chronic" series it was found among the more intense and in the "acute" series among the weaker antagonists. The difference in the results of the two series could be explained by the assumption that in the "chronic" experiments a metabolite with antagonistic action was formed from paludrine during the period of its administration.

At the end of the 30 min. period of continuous infusion in the "acute" experiments, the paludrine concentration in the blood was sometimes as high as 9 mg./l. In spite of the fact that such high drug concentrations could be obtained only a slight antagonism could be demonstrated. It seems likely that during the infusion period only a small proportion of the drug was metabolized into the hypothetical substance responsible for the antagonism.

It has also been suggested that paludrine itself does not possess plasmodicidal action when examined by a tissue culture method (Hawking, 1947; Hawking and Perry, 1948), but the serum of animals which have been treated with paludrine has a high plasmodicidal activity; from these results Hawking has deduced that the antimalarial activity of paludrine is not due to the drug itself but to some metabolite.

The formation of metabolites with biological actions in the guinea-pig different from those of paludrine has been demonstrated in the heart-lung experiments. In these preparations the addition of small amounts of mepacrine and quinine to the blood reduced the duration of the block produced when adenosine was injected into the venous cannula. If similar amounts of paludrine were added to the blood there was no reduction of the effects of adenosine, but substantial decrease of these effects was observed when blood from paludrine-treated guinea-pigs, possibly containing metabolites of the drug, circulated in the preparation (Table V).

This result cannot be explained by differences in the paludrine content of the blood from the treated guinea-pigs and that of the blood to which paludrine had been added. In some experiments these concentrations as measured by the method of Spinks and Tottey were the same for all practical purposes. (This method consists in the acid hydrolysis of the base, after its extraction with benzene, to *p*-chloroaniline and subsequent diazotization and coupling of the latter. It does not give any indication of the possible *in vivo* changes undergone in the side chain of paludrine, provided that they do not alter the solubilities and rate of hydrolysis to any great extent. How alterations in the benzene ring would affect these results is difficult to predict.)

It is interesting to note that in the acute experiments the antagonism shown by mepacrine was not permanent, and about 90 min. after the infusion of the drug the action of adenosine returned to normal. In heart-lung preparations the antagonistic action of mepacrine and quinine could be even more easily reversed by changing the blood containing drug for fresh normal blood. It appears that the antagonism to adenosine is due mainly to the drug which circulates in the blood and not to that which is fixed in the tissues.

Pamaquin and paludrine, or more probably some metabolite of it, behave with respect to adenosine in the same way as the rest of the antimalarial drugs assayed. This is in contrast to the failure of riboflavine to antagonize their action on *L. casei*, a property common to the other antimalarials assayed. Perhaps paludrine does not show such antagonism because under the *in vitro* conditions of the test its transformation into the substances responsible for its adenosine antagonism and antimalarial activity does not take place. One of the reasons which led Curd and Rose (1946d) to suggest that the antimalarial activity of paludrine was different from that of other types of antimalarials was this failure of riboflavine to antagonize it. In the light of the experimental evidence that paludrine can be converted in the blood of treated animals into some metabolite which does share with other antimalarials the property of antagonizing the action of adenosine, the case for such assumed difference in the mode of antimalarial action is somewhat weakened.

Summary

Antimalarial compounds antagonize the action of adenosine on the guinea-pig heart and on the hen caecum.

A parallelism between the antimalarial activity and the power to antagonize adenosine has been demonstrated in most series of compounds examined.

In heart-lung preparations it has been found that unlike the other antimalarials tested paludrine does not antagonize the action of adenosine. However, the blood of paludrine-treated guinea-pigs did antagonize this action. This is taken as evidence that paludrine is metabolized to another compound with different pharmacological properties.

The considerations which led to the testing of antimalarials as antagonists of adenosine are discussed.

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THE EFFECT OF HEPATECTOMY ON THE ACTION OF CERTAIN ANAESTHETICS IN RATS

BY

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To investigate the role of the liver in the detoxication of anaesthetics, two main methods are available. First, the liver may be damaged by some drug such as carbon tetrachloride; if the anaesthetic has a greater effect than before, it is probable that it is normally detoxicated by the liver. This method is open to at least two objections. In the first place, it is impossible accurately to assess the amount of liver damaged by this means; and further it is possible that the carbon tetrachloride has some action of its own on the central nervous system. The second method, which we have used, is to remove by operation a large proportion of the liver and observe whether the effect of the anaesthetic is increased. This method has the advantage that the amount of liver tissue removed can be estimated fairly accurately, and it is unlikely that the operation has any complicating side-effects. Higgins and Anderson (1931) described a method for partial hepatectomy in rats, and the high survival rate and quick recovery of the animals make it suitable for this type of investigation.

METHOD

Albino rats weighing between 150 and 300 g. were used. Each rat was given a preliminary dose of the drug to be tested in the following manner. Food was withheld overnight, and the following morning the drug was given to the animal by stomach-tube in a dose calculated according to body-weight. At frequent intervals the rat was laid on its side, and when it remained in this position it was considered to be asleep. As soon as it got to its feet it was considered awake, and the sleeping-time was thus determined. At least five days later partial hepatectomy was carried out by the method of Higgins and Anderson (1931). The animals withstood the operation well, the mortality being about 10 per cent, and by the third day after operation appeared perfectly normal. For 24 hours after operation they were fed on bread and milk and 20 per cent glucose. Thereafter they were given the normal laboratory diet. On a chosen day after hepatectomy, the rat was given the same dose of drug as before, and the sleeping-time again determined in the same way. The experiment was planned to give at least 10 results on each of the following days after hepatectomy: 6th–7th, 8th–9th, 10th, 12th, 15th, 17th–18th, 20th, 22nd, and 26th–28th. The mean sleeping-time of the rats on each of these days after

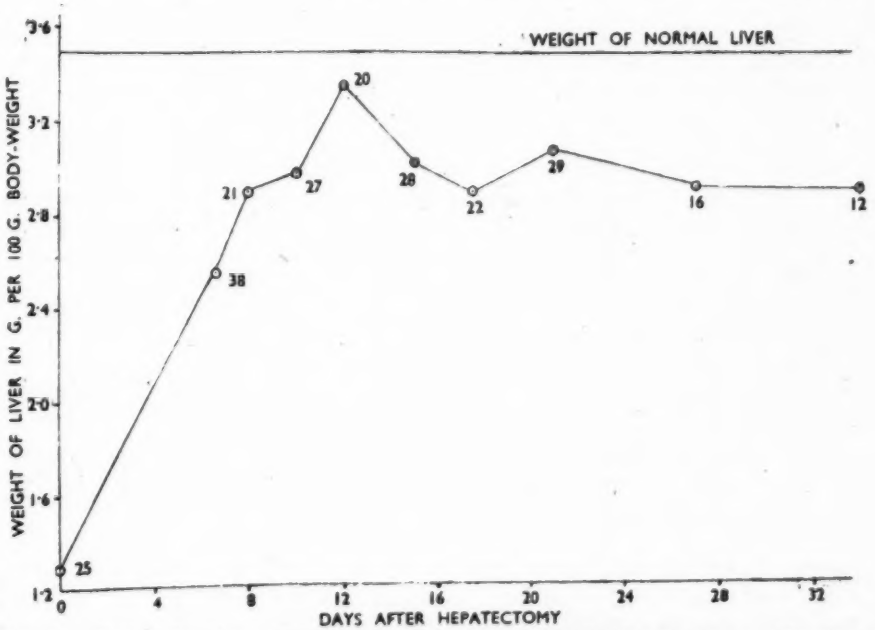

FIG. 1.—Graph showing regeneration of liver by weight after hepatectomy. Numbers at each point show the number of rats used to determine each mean.

operation could then be compared with their mean sleeping-time before operation.

Immediately each experiment was finished the rat was killed and its liver removed, washed in saline, dried with filter-paper, and weighed. No rat was used twice after hepatectomy.

The drugs and doses used were: chloral hydrate, 350 mg. per kg.; bromethol, 300 mg. per kg.; soluble phenobarbitone, 110 mg. per kg.; thiopentone, 50 mg. per kg.

Results

1. *Rate of regeneration of liver*

In order to obtain an average figure for the amount of liver removed at operation a preliminary experiment was carried out on 40 rats. The animals were hepatectomized and the portion of liver removed was weighed. The animals were then killed and the remainder of the liver was dissected out and weighed. From these two weights, the mean percentage of liver removed was calculated, and found to be 62.2 per cent by weight (S.D. = 4.88 per cent). This figure is lower than that of 70 per cent found by Higgins and Anderson (1931). Expressed as g. per 100 g. body-weight of rat, the mean weight of liver left behind after operation in these 40 rats was 1.29 (S.D. = 0.28) and the mean weight of the whole liver was 3.49 (S.D. = 0.81).

TABLE I

DIFFERENCES IN MEAN SLEEPING-TIME OF GROUPS OF RATS ON VARIOUS DAYS AFTER HEPATECTOMY. DEATHS SHOWN IN COLUMN (IV) ARE INCLUDED IN THE FIGURES IN COLUMN (V) EACH AS 500 MIN. SLEEPING-TIME

(i)	(ii)	(iii)	(iv)	(v)	(vi)
Number of rats	Day after hepatectomy	Mean sleeping-time in min. before hepatectomy	Number of deaths due to effect of drug after hepatectomy	Mean sleeping-time in min. after hepatectomy	Difference (v)–(iii)
Chloral Hydrate (350 mg. per kg.)					
16	6–7	171.1	4	346.1	+175.0
10	8–9	162.0	6	373.7	+211.7
10	10	85.2	4	277.4	+192.2
10	12	85.7	3	341.0	+255.3
10	15	209.4	7	384.0	+174.6
10	17–18	78.0	1	112.9	+34.9
10	22	176.3	0	74.8	−101.5
6	28	147.0	0	97.5	−49.5
Bromethol (300 mg. per kg.)					
16	6–7	59.7	8	416.6	+356.9
11	8	91.0	8	413.0	+322.0
10	10	77.5	6	396.6	+319.1
10	12	24.1	7	375.5	+351.4
10	15	111.7	4	281.6	+169.9
10	17–18	98.0	3	265.0	+167.0
10	22	76.7	0	93.5	+16.8
10	26	172.8	0	55.4	−117.4
Soluble Phenobarbitone (110 mg. per kg.)					
17	7–8	6.8	1	98.5	+91.7
10	10	0	0	78.1	+78.1
11	12	19.0	0	75.0	+56.0
10	16	0	0	20.5	+20.5
8	28	0	0	0	0
Thiopentone (50 mg. per kg.)					
3	4	133.3	2	573.3	+440.0
10	8	94.1	3	314.0	+219.9
8	12	12.0	2	227.0	+215.0
10	15	86.8	1	212.4	+125.6
8	20	92.0	1	110.0	+18.0

Fig. 1 shows the mean weights of the liver (in g. per 100 g. body-weight) of groups of rats on various days after hepatectomy. It will be seen that regeneration proceeded very rapidly during the first week after operation and that the liver had doubled its weight by the 7th day. In our experiments the liver had not regained its original weight by the 34th day, whereas in Higgins and Anderson's experiments it had regained its weight by the 14th day.

Brues, Drury, and Brues (1936) found that there was a fairly constant relationship between the weights of liver removed and that left behind, and therefore expressed the degree of liver regeneration as a percentage of the original liver weight. We have found that this method gives figures with a large scatter and that more consistent results are obtained by using the actual weight of liver in the rat in g. per 100 g. body-weight of rat.

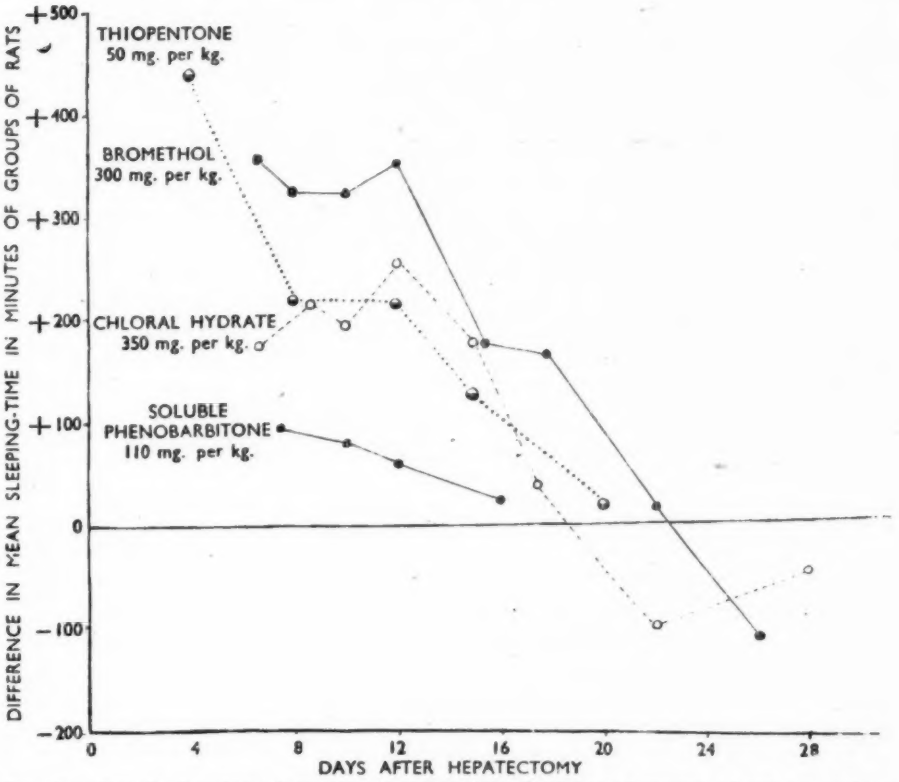

FIG. 2.—Graph showing differences in mean sleeping-times of groups of rats on various days after hepatectomy.

2. *Effect of anaesthetics after hepatectomy*

Table I shows the mean sleeping-times of groups of rats before and on various days after operation. Column (vi) gives the difference between these two figures and shows whether the effect of the drug was increased or not. In the early days after operation the greatly increased effect of the drug resulted in the death of some animals. In order to include these results in the calculations it was necessary to consider them as if they had slept for a long period. Since rats rarely slept after any of the drugs for more than 400 min., death was considered as equivalent to the arbitrary figure of 500 min. sleeping-time.

It can be seen from Table I that up to the 12th day after operation the effect of chloral hydrate was greatly increased. On the 18th day the effect was only slightly greater than that before operation, and on the 22nd day it was actually less. Similarly, Table I shows that by the 22nd day the effect of bromethol was almost the same as before, and was less than before operation on the 26th day. The results for soluble phenobarbitone show that there was a definite though smaller increase in sleeping-time after operation, and that this increase had almost disappeared by the 16th day. It will be seen that, with the dose of phenobarbitone used, animals rarely slept before operation. It was not possible to use a larger dose, which would produce sleep in most animals before operation, without causing a large number of deaths. Table I shows that by the 20th day the effect of thiopentone was almost the same as before hepatectomy. These results are expressed graphically in Fig. 2.

In order to make sure that the increased effect of these drugs after hepatectomy was due to the loss of liver substance and not to the operative interference or anaesthetic, control experiments were done for all four substances. A group of rats was given the usual dose of the drug; five days later laparotomy was carried out, and on the fourth day after operation a second dose of the

TABLE II

TABLE SHOWING THAT THERE IS NO CORRELATION BETWEEN DEGREE OF LIVER REGENERATION BY WEIGHT AND DIFFERENCE IN SLEEPING-TIME AFTER OPERATION. FIGURES ARE PERCENTAGES OF ANIMALS IN EACH GROUP

Drug	(i) Difference in sleeping-time in min.	(ii) Percentages of animals grouped in terms of weight of liver in g. per 100 g. body-weight <2.5g.	(iii) 2.5–3.0g.	(iv) 3.0–3.5g.	(v) >3.5g.
Chloral hydrate 350 mg./kg.	No increase	30	50	36	37
	+1–200	25	18	36	18
	+201–400	40	16	18	27
	+>400	5	16	10	18
Bromethol 300 mg./kg.	No increase	17	47	22	16
	+1–200	28	17	34	12
	+201–400	38	23	22	36
	+>400	17	13	22	36

drug was given as before. It was found that the increase in the mean sleeping-time was negligible.

We observed that rats before hepatectomy never slept for longer than 200 min. with any of the drugs. If the drug had a greater effect the animal died. After hepatectomy, however, the drug often caused rats to sleep for much longer periods. For instance, of 144 normal rats receiving bromethol, the highest sleeping-time was 190 min. Of 69 hepatectomized rats receiving bromethol, 10 slept for longer than 350 min. without dying. This observation is consistent with the idea that after hepatectomy the detoxication processes are slowed.

3. *Correlation of effect of drug with degree of liver regeneration*

We were interested to see if there were any correlation between the degree of regeneration of the liver and its ability to detoxicate anaesthetics. If there were a correlation, we should have expected animals with marked liver regeneration to be less affected by the anaesthetic than those whose livers had only regained a small proportion of their former weight. In Table II animals are arranged in four groups according to the weights of their livers at death (columns (ii), (iii), (iv), and (v)). Each of these groups is divided into four classes according to the difference in sleeping-time after hepatectomy. The figures in each column are the percentages of rats in each group. If there were a correlation, we should expect that a high percentage of animals with small liver-weights would have a much increased sleeping-time after operation, and conversely a high percentage of those with large liver-weights would have little or no increase in sleeping-time. It is clear from Table II that there is no such trend. For instance, for chloral hydrate, 45 per cent of rats whose liver weighed less than 2.5 g. per 100 g. showed an increased sleeping-time after operation of more than 200 min., whereas the same percentage (45) of animals whose livers weighed more than 3.5 g. also had an increase of more than 200 min. sleeping-time. Similarly, for bromethol, 55 per cent of animals with less than 2.5 g. liver per 100 g. and 72 per cent of animals with more than 3.5 g. per 100 g. showed an increase of sleeping-time after operation of more than 200 min. Hence the degree of regeneration of liver by weight is no guide to its ability to detoxicate these drugs.

Discussion

Chloral hydrate and bromethol are usually said to be detoxicated in the liver. Thus, Mukerji and Ghose (1940) found that, in dogs with livers damaged by carbon tetrachloride, there was a well-marked excretion of free chloral in the urine after oral administration of the drug, whereas in normal dogs all the urinary chloral was conjugated. There is, however, little definite evidence that bromethol is detoxicated in the liver, though it has been claimed that it can cause transient liver damage (Bourne and Raginsky, 1931; Coleman, 1938). Our experiments show that both these drugs are detoxicated in the liver, though the possibility that partial hepatectomy affects the renal excretion of them has yet to be excluded. Table II shows that there is no correlation between the degree of liver regeneration by weight and its ability to detoxicate the drug. It is clear that the restoration of the enzyme systems which break down or conjugate these drugs is independent of the restoration of the mass of liver tissue.

—Most observers state that phenobarbitone is not detoxicated in the liver. Cameron and De Saram (1939) found that there was no significant prolongation of its action in rats after acute liver damage caused by carbon tetrachloride. Masson and Beland (1945) stated that the anaesthetic effect of phenobarbitone was not increased by partial hepatectomy, whereas after bilateral nephrectomy rats slept for over twice as long as the controls. Similarly, Hirschfelder and Haury (1933) found that bilateral nephrectomy markedly increased the effect of phenobarbitone in rabbits. These and other workers, therefore, consider that soluble phenobarbitone is eliminated through the kidney and not detoxicated to a significant extent by the liver. It is, however, not possible to recover more

than 25 per cent of administered phenobarbitone in human urine (Halberkann and Reiche, 1927), so that 75 per cent is still to be accounted for. Our experiments show that partial hepatectomy increases the effect of soluble phenobarbitone up to the 16th day after operation. The possibility that hepatectomy affects the renal excretion of the drug in some way has yet to be excluded, but a probable explanation is that the drug is detoxicated to some extent in the liver.

It will be seen from Table I that the increase in mean sleeping-time with phenobarbitone on the seventh day after operation (90 min.) is much less than that with chloral and bromethol. This is possibly due to the relatively smaller dose of phenobarbitone used.

The evidence about thiopentone is conflicting. Richards and Appel (1941) found that its effect in rats was not increased after liver damage by carbon tetrachloride, and Scheifley (1946) found that its effect was not increased by partial hepatectomy. On the other hand, Schideman, Kelly, and Adams (1947) found that partial hepatectomy, liver damage with carbon tetrachloride, and the production of an Eck fistula all markedly increased the effect of thiopentone. They also showed that the drug was broken down when incubated *in vitro* with liver slices. Our results support these authors' conclusions that thiopentone is detoxicated in the liver.

Fig. 2 shows that between the 22nd and 26th day the liver appears to be better able to detoxicate chloral hydrate and bromethol than before hepatectomy. Further work is necessary to confirm and elucidate this phenomenon. It is impossible to say whether the same holds good for phenobarbitone, since, with the dose used, animals seldom sleep before operation.

Summary

1. A method is described of determining whether anaesthetics are detoxicated in the liver, by using partial hepatectomy in rats.

2. The anaesthetic effect of chloral hydrate, bromethol, soluble phenobarbitone, and thiopentone is increased by partial hepatectomy. This increased effect is no longer present for chloral hydrate by the 17th, for bromethol by the 22nd, for soluble phenobarbitone by the 16th, and for thiopentone by the 20th, day after operation.

3. There is no apparent correlation between the regeneration of the liver by weight and its power to detoxicate these drugs.

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THE PHARMACOLOGY OF THE OPTICAL ISOMERS OF AMIDONE (2-DIMETHYLAMINO-4 : 4-DIPHENYLHEPTAN-5-ONE)

BY

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A brief description of the preparation of the optical isomers of amidone has already been published (Thorp *et al.*, 1947b), in which it was shown that it is possible to prepare these substances by resolution of 1 : 1 - diphenyl - 3 - dimethylamino-valeronitrile, the penultimate compound in the synthesis of amidone, and conversion of the optically active nitriles into the corresponding ketones. Some brief pharmacological results were given in that note, but it is now possible to describe the pharmacology of these substances more fully.

Analgesic activity

The relative analgesic activities of *d*-, *l*-, and *dl*-amidone were determined in comparison with that of morphine. The method used has already been described by the author (1946) ; it was a modification of the radiant heat stimulation method of Hardy, Wolff, and Goodell (1940). The comparison was performed on a group of forty rats on each of four days, and two dose levels for each drug were used. The animals were randomly arranged into eight groups of five rats, and the treatments changed on each day so that all rats had received one dose of each drug at the completion of the test. The initial pain threshold was determined before injection ; the drugs, dissolved in amounts of saline such that the appropriate doses were contained in 0.5 ml. per 100 g. of rat, were injected subcutaneously at intervals of 2 min. This interval was introduced in order to allow for the time taken in estimating the pain threshold subsequent to injection, so that responses might be measured at equal intervals.

Previous tests had indicated that the effects of morphine and amidone upon the pain threshold of rats are maximal approximately 30 min. after injection and that the curves for the duration of action of the two substances are almost identical. A single determination of the pain threshold half an hour after injection was therefore used for estimations of analgesic potency.

Table I shows the results obtained in the comparison of the analgesic activities of *d*-, *l*-, and *dl*-amidone with that of morphine.

The equiactive doses in the sixth column were obtained by estimating the dose of each drug producing a 50 per cent increase in pain threshold,

TABLE I

THE COMPARATIVE ANALGESIC ACTIVITIES OF THE OPTICAL ISOMERS OF AMIDONE BY THE RAT METHOD

Drug tested	Dose mg./kg.	No. of rats	Mean increase in pain threshold			Equiactive doses mg./kg. (±%S.E.)	Activity ratio (morphine = 1)
			Per cent	Standard error	Slope		
d-Amidone hydrochloride	10 20	20 20	34.9 34.8	3.45 3.65	nil	—	—
l-Amidone hydrochloride	0.8 1.2	20 20	31.9 61.8	3.19 5.75	169.8	1.02 ±10.45%	2.1
dl-Amidone hydrochloride	1.2 2.4	20 20	41.0 63.5	3.78 8.80	74.8	1.58 ±12.05%	1.36
Morphine sulphate	2.0 3.0	20 20	46.8 63.7	4.72 10.01	96.0	2.16 ±11.15%	1.0

the slope value estimated for each regression line being used.

The activity ratios are the ratios of the reciprocals of the equiactive doses taking the value for morphine as unity. The three values for the slopes were tested for similarity, and it can be shown that, for $P = 0.95$, the differences between them are not statistically significant. The standard errors of the equiactive doses in the fifth column do not preclude the possibility that *l*-amidone is twice as active as the racemic isomer.

The results show that in the rat *dl*-amidone hydrochloride is probably a rather more powerful analgesic drug than morphine sulphate, and that the activity of the *laevo* isomer is very much greater than that of the racemic form.

The *dextro* isomer failed to produce a graded increase in pain threshold when given in large doses, and the observed rise in pain threshold was probably not due to true analgesic action. In doses comparable with those of *dl*-amidone, *d*-amidone is without effect upon the pain threshold in rats. It is therefore very probable that the *laevo* isomer is twice as active as the racemic form and the *dextro* compound inactive.

Toxicity

The toxicity of the optical isomers of amidone was determined by intravenous injection into mice, the method of calculating the LD50 value described by Kärber (1931) being used. The doses were always given in 0.25 ml. of physiological saline and the results obtained are given in Table II.

It will be seen that the LD50 values for the three isomers are of the same order of magnitude. The actual values obtained do not, however, agree with those previously reported by the author (1947a ; 1947b), which were between 10 and 18 mg./kg. We have repeated acute toxicity experiments on mice of two different strains and upon different days, and found that the absolute values of the LD50 for amidone isomers are subject to wide variations, although the results are comparable within individual experiments.

Finnegan *et al.* (1948) found the LD50 of *dl*-amidone hydrochloride to be 9.2 ± 0.4 mg./kg. in rats upon intravenous injection, but pointed out that the toxicity in acute experiments is very much greater than that of morphine, whereas the values obtained with smaller doses in a subacute experiment were much more similar.

These findings show that the acute toxicity of amidone is not due to the central nervous depressant property since this is almost absent in the *dextro* isomer. The acute toxic effect is the result of sudden cardiovascular failure, and the LD50 value is determined largely by the resistance of the animals to a critical fall of blood pressure of brief duration

There is evidence, however, from the results given above that the toxicity of *l*- and *dl*-amidone is enhanced by the depressant action these drugs exhibit since the LD50 value for *d*-amidone is just significantly greater than that of the other isomers.

When cats and dogs were anaesthetized with pentobarbitone sodium and arranged for recording blood pressure and respiration, the following results were obtained.

d-*Amidone.*—In both dogs and cats similar effects were observed after intravenous injections of *d*-amidone. Small doses of 1.0 or 2.0 mg./kg. caused a brief fall in blood pressure of 40–80

TABLE II

THE ACUTE TOXICITIES OF THE OPTICAL ISOMERS OF AMIDONE UPON INTRAVENOUS INJECTION INTO MICE

d-Amidone HCl		*l*-Amidone HCl		*dl*-Amidone HCl	
Dose mg./kg.	Mortality	Dose mg./kg.	Mortality	Dose mg./kg.	Mortality
22.64	0/20	11.32	0/20	8.00	0/20
32.00	14/20	16.00	2/20	11.32	2/20
45.28	20/20	22.64	9/20	16.00	3/20
		32.00	14/20	22.64	8/20
		45.28	17/20	32.00	14/20
		64.00	20/20	45.28	20/20
LD50 = 29.9 mg./kg. Fiducial limits (P = 0.95) 27.47 to 32.58		LD50 = 26.02 mg./kg. Fiducial limits (P = 0.95) 22.89 to 29.55		LD50 = 24.24 mg./kg. Fiducial limits (P = 0.95) 21.16 to 27.76	

mm. Hg. There was no depression of the respiration and when a series of doses of 2 mg./kg. were given at 15-min. intervals a similar fall in blood pressure was produced with each injection. The duration of each depressor response was from 1 to 2 min. Death resulted in all animals after the injection of 7–10 mg./kg. of *d*-amidone and was characterized by a precipitate fall of blood pressure to approximately one-third of the normal level. This effect was associated with temporary respiratory arrest followed by Cheyne - Stokes respiration, and death usually occurred in 20 min. or less. These effects were not modified by section of both vagi in dogs.

When guinea-pigs in which the brain and upper part of the spinal cord had been destroyed were supplied with artificial respiration and arranged for recording blood pressure, injections of 1 mg./kg. of *d*-amidone caused a marked fall in blood pressure similar to that observed in anaesthetized dogs or guinea-pigs.

These experiments show that the principal cause of the toxic action of *d*-amidone is a precipitate fall in blood pressure which is due to direct action upon the cardiovascular system.

l-*Amidone.*—The injection of *l*-amidone into the anaesthetized dog or cat caused effects which were markedly different from those described above. In the dog a first injection of 1 mg./kg. of *l*-amidone caused a fall of blood pressure of approximately 50 mm. Hg lasting about 1½ min. The blood pressure then rose to a value slightly above the normal. The respiration showed marked depression and became irregular. A second dose of 1 mg./kg. caused a much smaller fall in blood pressure followed almost at once by a prolonged increase of approximately 30 mm. Hg above the normal level. The respiration showed a further degree of depression. A third similar dose 10 min. later caused no detectable fall in blood pressure, but a further rise in the general level. After both vagus nerves were cut and the animal left for an interval of an hour, injections of 1 and 2 mg./kg. of *l*-amidone caused only a fall in blood pressure.

In the cat the blood pressure showed the same brief fall upon injection of the drug, followed by a return to a level slightly below the normal value. Subsequent doses caused a progressively less marked initial fall, but a general lowering of the level of blood pressure followed.

This initial fall in blood pressure has been examined by Shideman and Johnson (1948), who have shown that it becomes less upon subsequent injections and is a phenomenon exhibited by most analgesic drugs. They have termed the effect "acute vascular tolerance."

The effects of *l*-amidone were in general indistinguishable from those of *dl*-amidone described by Scott and Chen (1946), and, in our experiments upon animals in which the vagi were intact, cardiac slowing was also observed after injections of *l*-amidone.

The acute effects of the amidone isomers appear to be due to two principal actions: an immediate fall in blood pressure caused by direct action upon the heart and possibly also the vascular system, and, with the *l*- or *dl*-isomers, an additional depression of respiration and some slight degree of parasympathetic stimulation.

Effect upon respiration

The effect upon respiration was compared in the rabbit by means of the respiration recorder described by Gaddum (1941). The recorder was

TABLE III

THE EFFECT OF THE OPTICAL ISOMERS OF AMIDONE UPON THE RESPIRATORY MINUTE VOLUME IN RABBITS

d-Amidone HCl			*l*-Amidone HCl			*dl*-Amidone HCl		
Dose mg./kg.	Max. change in respiration per cent	Mean	Dose mg./kg.	Max. change in respiration per cent	Mean	Dose mg./kg.	Max. change in respiration per cent	Mean
1.5	+15	+19.5	1.4	−46	−37.3	2.8	−55	−56.5
1.5	+24		1.4	−32		2.8	−64	
2.5	+3		1.4	−34		2.8	−60	
2.5	−10	+4.7	1.7	−47	−51.2	2.8	−47	
2.5	+14		1.7	−53		3.4	−66	
2.5	+12		1.7	−53		3.4	−55	−61.5
5.0	−5	−5.0	2.5	−87	−89.0	3.4	−47	
			2.5	−91		3.4	−78	
						5.0	−94	−95.0
						5.0	−96	

connected to a rubber mask with inspiratory and expiratory valves and fitted to unanaesthetized rabbits restrained in a prone posture by broad cotton tapes. Since analgesic compounds commonly cause a marked fall in body temperature the animals were placed in a ventilated air thermostat maintained at 24° C. The recorder was of the differential type described in Gaddum's paper with tambours 4 in. in diameter. In these circumstances the record obtained was linearly proportional to the respiratory minute volume from a recorded height of 2 to 12.5 cm. upon the kymograph. Four to eight rabbits were used for the comparison of each compound with morphine as the "standard," and the peak percentage of respiratory depression was measured.

Both *dl*- and *l*-amidone depress the rabbit respiration to a marked degree when doses are given slightly in excess of those required to produce analgesia.

Table III shows the percentage change of the normal respiratory minute volume of rabbits measured at the point of maximum respiratory depression on the continuous records obtained with Gaddum's respiration recorder.

The results are expressed graphically in Fig. 1, from which it will be seen that the depressant effect of *l*-amidone upon the rabbit respiration is twice as great as that of the racemic compound, the effect of the *dextro* isomer being negligible in comparable doses. Some degree of stimulation is possibly caused by the latter drug.

The respiratory depressant property parallels the occurrence of analgesic properties in these three compounds and is therefore not the reason for the high toxicity of all three isomers.

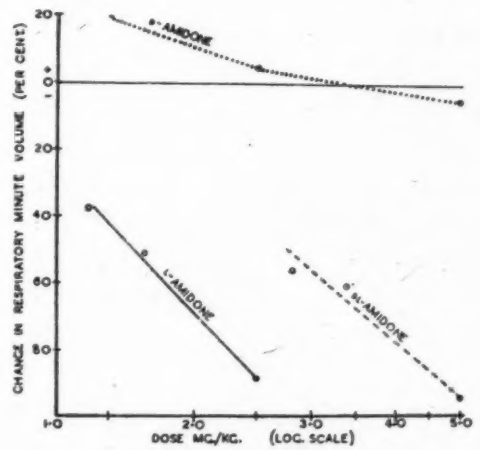

FIG. 1.—The effect of the optical isomers of amidone upon the respiratory minute volume of rabbits.

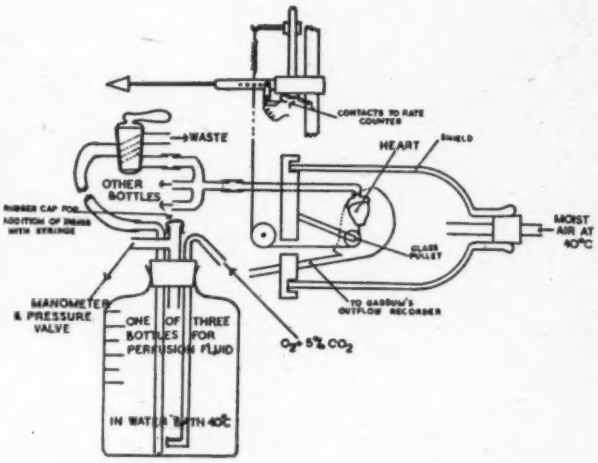

FIG. 2.—Apparatus for the perfusion of the isolated rabbit heart which enables the effect of accurately measured concentrations of drugs to be investigated.

Effect upon the isolated rabbit heart

The effect upon the isolated rabbit heart was estimated by means of the Langendorff preparation, but a modified apparatus was used which enabled the heart to be exposed to accurately known concentrations of the drugs.

This apparatus is shown diagrammatically in Fig. 2 and consists of a rigid vertical board through which the aortic cannula is mounted. This cannula can be connected to any one of three bottles of 1 litre capacity by three two-way stopcocks. The bottles, filled with Ringer solution, are kept at 39° C. in a water-bath. By this means the drug under test can be administered in known dilution from one of the bottles, and, after the rubber tubing connecting the cannula has been flushed out by means of the two-way tap, this solution can be substituted for the Ringer solution already flowing through the heart.

The outflow from the coronary vessels is recorded by means of a funnel beneath the heart carrying the perfusate to a Gaddum outflow recorder. The heartbeat is recorded by a thread from the apex of the left ventricle passing over a glass pulley and through a hole in the board to a lever on the kymograph. A record of the heart rate is made simultaneously by counting the closing of a pair of springy contacts mounted at the hinge of the recording lever. These contacts connect to a valve relay, and an impulse counter, described by the author (1948), is used to make the final record. By this means changes in coronary flow, heart rate, and the amplitude of contraction with each beat are recorded simultaneously.

The data shown in Table IV were obtained by means of the method described above. Results

TABLE IV

THE EFFECT OF THE OPTICAL ISOMERS OF AMIDONE AND OTHER ANALGESIC DRUGS UPON THE LANGENDORFF PREPARATION OF THE ISOLATED RABBIT HEART

Drug	Dilution	Effect upon the isolated rabbit heart			
		Coronary flow	Heart rate	Amplitude of beat	Notes
d-Amidone HCl	1 : 500,000 1 : 100,000	Nil +25%	Nil Slowed	Decreased Greatly decreased	Toxic conc.
l-Amidone HCl	1 : 500,000 1 : 100,000	+20% +30%	Nil Slight slowing	Decreased Greatly decreased	Toxic conc.
dl-Amidone HCl	1 : 500,000 1 : 100,000	+10% +20%	Nil Slowed	Decreased Greatly decreased	Toxic conc.
Morphine sulphate ..	1 : 100,000 1 : 50,000	Nil Nil	Nil Nil	Nil Nil	Non-toxic
Pethidine ..	1 : 100,000 1 : 20,000	Nil +10%	Nil Nil	Decreased Greatly decreased	Toxic conc.

obtained with morphine and pethidine were included for comparison.

It will be seen that all three isomers of amidone behaved similarly upon the heart. The toxic level was similar with each isomer and was found at concentrations of 1 : 100,000 and stronger. If it be assumed that the drug is distributed uniformly upon intravenous injection this level corresponds to a dose of the order of 10 mg./kg., a level comparable with that expected from the toxicity determinations.

Pethidine also proved to be toxic to the heart, but the concentration required was five times as great as that of amidone isomers ; this finding again agrees with the acute toxicity ratios for these drugs. Morphine, which is of low toxicity in mice and rats, was without cardiotoxic action at concentrations of 1 : 20,000 and less.

The principal toxic action of amidone isomers is therefore upon cardiac muscle, but it is increased with the *l*- and *dl*-isomers by the central nervous depression which these two drugs produce. The apparent stimulation of respiration shown by *d*-amidone is probably real and secondary to the fall of blood pressure produced by the action upon the heart of small doses.

Spasmolytic activity

Segments of rabbit ileum, caused to contract with a concentration of 1 in 4,000,000 carbamylcholine, showed approximately 70 per cent relaxation of the spasm after the addition of any one of the three amidone isomers in a concentration of 1 in 150,000 in the isolated organ bath. When 1 in 5,000 barium chloride was used in the same way each of the amidone isomers caused similar relaxation in a concentration of 1 in 120,000.

Pethidine was tested in the same way for purposes of comparison ; after carbamylcholine it had a similar spasmolytic effect in a concentration of 1 in 70,000 and after barium chloride the same concentration was effective.

These results show that amidone and its optical isomers are rather more active as spasmolytic drugs than pethidine. This effect is not associated with the optically active carbon atom but is a function of the molecule as a whole ; the action on the isolated heart already described is equally independent of the stereochemical configuration. Probably they act directly upon the muscle cells.

Local anaesthetic action

The local anaesthetic activity of the three isomers was compared with that of procaine by the intracutaneous weal method described by Bülbring and Wajda (1945). Eighteen to 24 guinea-pigs were used for each drug with two intracutaneous weals on each animal. Each animal received one weal from procaine and one weal from one of the test compounds. In half of the group of guinea-pigs high doses of procaine or of the amidone isomer were placed anteriorly and low doses posteriorly, and in the other animals the arrangement was reversed ; this was done in order to compensate for the slightly greater sensitivity of the skin in the anterior half of the body.

The results were assessed by calculating the mean slope of the dose response curves for

procaine and the amidone isomers, and thereby obtaining the potency ratio for the various drugs. A weighted mean potency ratio was then calculated for the group of animals treated with the same compound.

TABLE V

A COMPARISON OF THE LOCAL ANAESTHETIC PROPERTIES OF THE OPTICAL ISOMERS OF AMIDONE USING THE METHOD OF BÜLBRING AND WAJDA

The volume of solution injected for each dose was 0.1 ml.

Drug	No. animals	Conc. of drug mg./ml.	Mean potency ratio (procaine = 1)
d-Amidone hydrochloride	18	4.0 2.0	0.58
l-Amidone hydrochloride	24	2.0 1.0	3.51
dl-Amidone hydrochloride	24	2.0 1.0	2.14

The results are given in Table V from which it will be seen that local anaesthetic action is shown by all three isomers and is greatest in the *laevo* form. The potency of the racemic compound is the mean of those of the *d*- and *l*-isomers. Consequently the optical isomerism of amidone greatly influences the local anaesthetic activity, which is 3.5 times as great as procaine for the *laevo* isomer, but that this is not the only factor influencing this activity is shown by its occurrence with all three isomers.

Effect upon body temperature

The effect of these amidone isomers upon body temperature was determined in rabbits, six animals being used for each experiment. The temperatures were measured by rectal thermocouples connected to a Tinsley amplifier through a selector switch connecting each rabbit to the recorder once in 2 min. The final record was taken by means of a Siemen's six-colour chart recorder. The doses were given subcutaneously in a volume of 1 ml. physiological saline.

Groups of six rabbits were used together at one time, but each drug was given to more than one such group since some rabbits occasionally showed no effect at all from the drug. This absence of effect has previously been recognized in rabbits being used for testing pyrogenic substances, and a preliminary sorting test is usually employed to select animals of comparable sensitivity. Rabbits which showed no effect with the active drugs in these experiments were not included in the results. The remaining animals tended to fall into two groups, the larger one of which exhibited consistent changes in body temperature to the same treatment and a second smaller group of very sensitive animals which showed a very much more marked depression of temperature. These more sensitive animals numbered about 15 per cent of the total used. For the purposes of these comparisons all animals showing a fall in temperature were used for the calculation of the mean curves reproduced in Fig. 3.

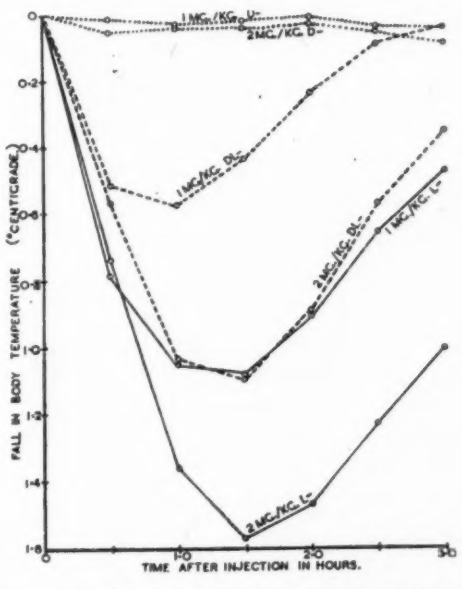

FIG. 3.—The effect of optical isomers of amidone upon the body temperature of rabbits.

The curves show that *d*-amidone had no effect upon body temperature, *l*-amidone produced the most marked depression, and the racemic isomer produced an effect which was very nearly half that of the *l*-isomer.

DISCUSSION

The central depressant actions of amidone are associated only with the *laevo* isomer. Consequently the racemic compound is approximately half as active on the central nervous system. The effects of amidone are complicated by the fact that some structural features other than the stereochemical configuration confer on the molecule marked toxic properties to the muscle cell, since these are also displayed by the *dextro* isomer.

The action of amidone upon the cardiovascular system has been examined by Scott and his colleagues (1947) using the cross-circulation technique of Heymans in dogs. They have shown that

the direct hypotensive effect was confined to the donor dog and was not the result of vagal mediation, and they concluded, because of the slowing of the heart of the recipient dog, that amidone was a stimulant of central parasympathetic nuclei, although the drug does not show any other characteristic parasympathetic effects.

The acute toxicity of amidone is almost entirely due to the direct effect on the heart whereas this type of effect is very small with morphine; this explains the findings of Finnegan *et al.* (1948) that morphine is far less toxic in acute experiments but that in chronic experiments with smaller doses there is less difference between this drug and amidone.

Compounds of the amidone type commonly show powerful local anaesthetic activity, and it is curious that among the effects which are influenced by the stereochemical configuration this was the only one in which the *dextro* isomer had a considerable degree of activity.

Summary

1. The *dextro, laevo,* and racemic optical isomers of amidone (2-dimethylamino-4 : 4-diphenylheptan-5-one) have been examined pharmacologically. The effects upon the central nervous system in mammals are associated with the *laevo,* and consequently also the racemic form.

2. The site of action of the acute toxicity of amidone was found to be upon the cardiac muscle cells. All three isomers of amidone were approximately equally toxic.

3. Spasmolytic activity was shown to be a function of the general structure of amidone and not associated with optical isomerism.

4. Local anaesthetic activity occurs in all three isomers, but is influenced by optical isomerism and is greatest in the *laevo* form.

5. The recently reported property of analgesic drugs, of producing a state of "acute vascular tolerance" to the depressor action resulting from intravenous injection, has been confirmed with *l*-amidone.

My thanks are due to Messrs. J. B. Attlesey, D. R. Howard, and R. F. Sellar for technical assistance. In addition I should like to thank Dr. E. Walton, of the Wellcome Chemical Research Laboratories, and Mr. P. Ofner, of the Wellcome Chemical Works, for the separation and provision of supplies of the optical isomers of amidone.

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ANTIMITOTIC ACTION OF MALEIMIDE AND RELATED SUBSTANCES

BY

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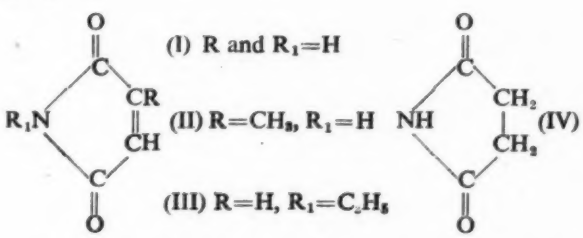
The results obtained by us (1948a) with maleic acid as an inhibitor of mitosis have been developed in an attempt to prepare other mitotic inhibitors related to maleic acid ; in particular we have investigated the antimitotic activity of substances in which the maleic acid residue is part of an aliphatic ring. The imides of maleic acid (I) and of citraconic acid (II), N-ethylmaleimide (III), and in addition succinimide (IV) were chosen for this purpose.



The action of these substances on the growth of normal cells is reported in this paper.

METHODS

The experiments were carried out on tissue cultures of chick fibroblasts. The technique adopted by us has been described previously (1948a). The *hanging drop method* was used in all experiments except some with maleimide, in which the Carrel flask method was adopted in combination with the Kodak record film technique.

Carrel flask technique.—By this technique the tissues are grown in a solid coagulum of blood plasma at the bottom of a small flask. The embryo extract containing the compound is added as soon as the plasma has clotted.

Cine micrographical record.—A picture was taken every sixth minute on a Kodak recording film. The mitotic index was assessed by counting the mitoses occurring in each photograph and by making a total cell count at the end of every tenth hour. The percentage of hourly mitoses was calculated in terms of the number of cells present. Details of this technique have been described by Willmer and Jacoby (1936).

EXPERIMENTAL

Mitotic Disturbances

The values for mitotic inhibition and phase distribution obtained with compounds I, II, III, and IV are collected in the Table.

Maleimide (I ; R and R_1 = H)

Maleimide has weak antimitotic activity. At $5 \times 10^{-6}M$ an inhibition of 21 per cent was observed. By raising the concentration to $9 \times 10^{-6}M$ no further increase of the inhibition has been observed.

At lower concentrations of maleimide 1×10^{-6} M (Table) and occasionally at $3 \times 10^{-6}M$, an increase in the mitotic count occurred after 1.5, 3, and 24 hours (Fig. 1). A number of experiments was performed to ascertain the nature of

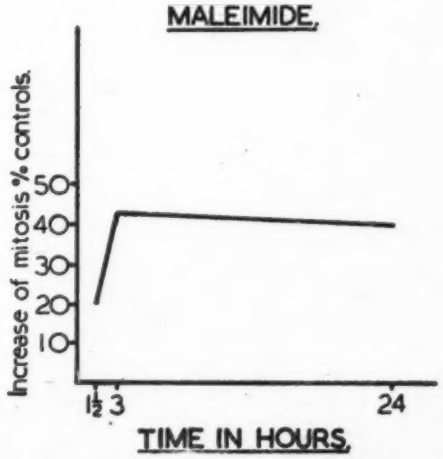


FIG. 1.—Increase in mitotic count with $3 \times 10^{-6}M$ maleimide at 1.5, 3, and 24 hours.

TABLE

TISSUE CULTURES: CHICKEN FIBROBLASTS, HANGING DROP METHOD, 4TH PASSAGE, 24-HOUR CULTURES, FIXED IN SUSA, STAINED IN HEIDENHAIN'S HAEMATOXYLIN

Exp.	Molar conc.	Mitoses as % of mitoses of controls	Per cent inhibition	Phase distribution in % of mitoses							
				Prophase		Metaphase		Anaphase		Telophase	
MALEIMIDE (9,619 mitotic cells investigated)											
1	Controls	—	—	15.4		47.2		6.2		31.2	
2	1×10^{-6}	128.4 ± 7.9%	0		14.0		34.0		6.8		45.2
3	3×10^{-6}	99.0 ± 5.4%	0		19.0		25.0		3.0		53.6
4	Controls	—	—	12.7		39.1		5.7		42.5	
5	5×10^{-6}	79.0 ± 6.1%	21.0		22.2		29.3		2.1		46.4
6	7×10^{-6}	76.7 ± 8.2%	23.3		16.8		30.3		4.9		48.0
7	9×10^{-6}	79.0 ± 4.8%	21.0		28.3		28.9		4.9		37.9
8	Controls	—	—	16.8		30.9		3.6		48.7	
9	3×10^{-6}	139.0 ± 1.3%	0		17.2		22.0		3.4		57.4
CITRACONIMIDE (4,634 mitotic cells investigated)											
1	Controls	—	—	23.4		20.8		4.3		51.5	
2	3×10^{-6}	38.2 ± 2.8%	61.8		19.5		31.6		4.5		44.4
3	Controls	—	—	10.0		45.6		6.5		37.9	
4	1×10^{-6}	66.7 ± 2.1%	33.3		6.3		34.9		13.6		45.2
5	6×10^{-7}	80.6 ± 3.2%	19.4		14.1		43.1		6.8		36.0
6	4×10^{-7}	89.8 ± 4.9%	10.2		7.5		42.8		9.4		40.3
N-ETHYLMALEIMIDE (5,070 mitotic cells investigated)											
1	Controls	—	—	22.1		31.8		1.6		44.5	
2	4×10^{-7}	82.0 ± 2.9%	18.0		16.3		36.1		5.1		42.5
3	6×10^{-7}	72.7 ± 3.7%	27.3		9.1		39.5		11.3		40.1
4	1×10^{-6}	67.9 ± 3.0%	32.1		19.5		34.9		5.9		39.7
5	Controls	—	—	12.2		40.8		8.6		38.4	
6	2×10^{-6}	49.2 ± 2.4%	50.8		10.7		45.4		1.6		42.3
7	4×10^{-6}	52.9 ± 1.9%	47.1		10.5		43.2		5.2		41.1
8	6×10^{-6}	47.9 ± 2.3%	52.1		16.1		41.1		3.7		39.1
SUCCINIMIDE (5,373 mitotic cells investigated)											
1	Controls	—	—	18.8		27.0		6.2		48.0	
2	3×10^{-6}	97.6 ± 3.5%	2.4		19.1		30.2		6.1		44.6
3	Controls	—	—	15.6		27.1		3.4		53.9	
4	5×10^{-6}	84.3 ± 3.4%	15.7		17.8		41.2		2.6		38.4
5	7×10^{-6}	96.5 ± 3.0%	3.5		15.0		41.0		2.1		41.9
6	9×10^{-6}	93.6 ± 2.1%	6.4		17.3		40.0		1.8		40.9

this increase. The Carrel flask method in combination with the Kodak record film technique made it possible to count the mitoses hourly in unstained preparations, whereas in the hanging drop method stained cells are counted only after 24 hours. The film showed that some cells remained in metaphase for 8–12 hours and that other cells never completed cell division. In the 24-hour period used in the hanging drop method, therefore, not only the cells which are going into division are counted but in addition those which persist in metaphase. Thus the Kodak record film technique gave clear evidence that the increase of the mitotic count after 24 hours consisted in an accumulation of metaphases.

Abnormal cells were found at all concentrations; they were confined mostly to clumped metaphases. Enlarged and vacuolated cells were

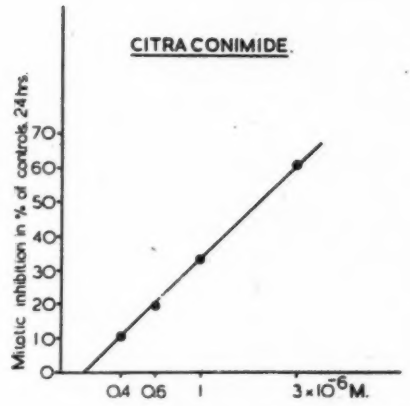


FIG. 2.—Mitotic inhibition plotted as percentage of controls against the logarithm of the concentration of citraconimide.

present. At $1 \times 10^{-6}M$, the lowest concentration investigated, a few tripolar cells (3 in 470 metaphases), fragmented chromosomes, and chromosome bridges (10 in 142 anaphases) were observed.

Citraconimide (II ; R = CH_3, R_1 = H)

Citraconimide had the strongest antimitotic properties of the substances of this group. The mitotic inhibition increased with rising concentration. At $3 \times 10^{-6}M$ the inhibition was 61.8 per cent. The increase followed closely a logarithmic graph when plotted against the $\log_{10}$ of the concentrations (Fig. 2). Microscopically no abnormalities were found in significant quantities.

N-ethylmaleimide (III ; R = H, $R_1 = C_2H_5$)

N-ethylmaleimide showed increasing antimitotic activity from $0.4 \times 10^{-6}M$ to $2 \times 10^{-6}M$; at the latter concentration 50.8 per cent inhibition was observed. A further rise in concentration did not lead to greater inhibition. Microscopically, no abnormalities in significant numbers are present.

Succinimide (IV)

Succinimide showed no mitotic inhibition and no abnormal cells were observed.

Sulphydryl Uptake

In a previous paper (1948b) we drew attention to a parallelism between mitotic inhibition and –SH uptake, which was clearly evident in the maleic acid series. In order to ascertain whether a similar parallelism could be established in the maleimide series, we investigated the –SH uptake of maleimide, citraconimide, and of N-ethylmaleimide, using thiolacetic acid and glutathione as –SH donors and following the directions given by Morgan and Friedmann (1938) for maleic acid and these two –SH compounds.

At room temperature and at a final concentration of $M/50$ for each component, maleimide took up 100 per cent of thiolacetic acid and 100 per cent of glutathione within 2 min. Citraconimide took up 70 per cent of thiolacetic acid and 75 per cent of glutathione within 3 min. After this quick uptake the reaction slowed down and the curves representing it become asymptotic. N-ethylmaleimide behaved like maleimide and showed 100 per cent –SH uptake from thiolacetic acid and glutathione within 1 min.

Fig. 3 demonstrates the –SH uptake from glutathione as –SH donor with maleimide and citraconimide as –SH acceptors at room temperature.

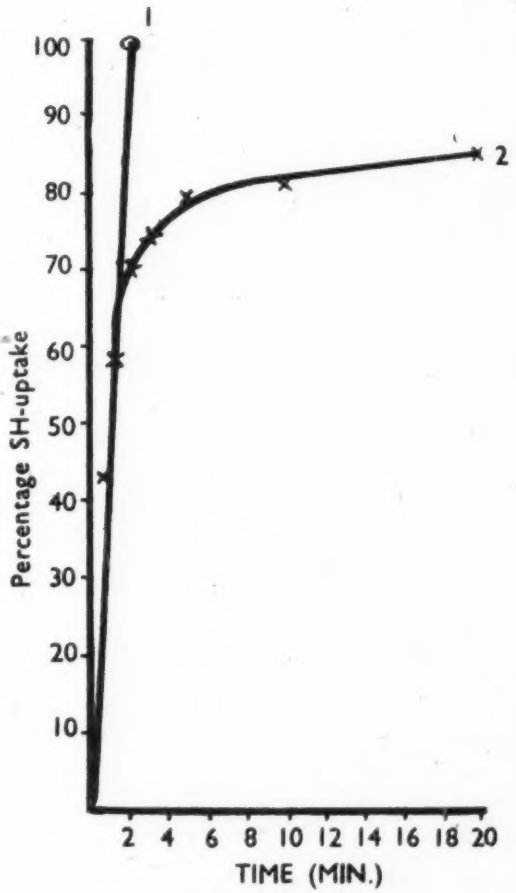


FIG. 3.—SH uptake of maleimide and citraconimide. Curve 1: maleimide and glutathione, final concentration $M/50$. Curve 2: citraconimide and glutathione, final concentration $M/50$.

Discussion

Of the four substances investigated, three are unsaturated and only one, succinimide, is saturated. The three unsaturated substances, maleimide, citraconimide, and N-ethylmaleimide, have antimitotic properties, whereas the saturated compound, succinimide, is devoid of antimitotic activity. These mitotic inhibitors share with the other mitotic inhibitors, encountered previously—i.e., maleic acid and the 1 : 4-naphthoquinones —the property of forming –SH adducts.

The –SH uptake of the maleimides is extremely rapid compared with the –SH addition to maleic acid: $M/25$ maleic acid needs 6 hours for a 50 per cent –SH uptake from $M/50$ thiolacetic acid and $M/50$ glutathione, whereas maleimide and N-ethylmaleimide accomplish a 100 per cent –SH uptake from the same substances in 1–2 min. and citraconimide a corresponding uptake of 70 per cent in 3 min. It will be seen that the introduction of a methyl group in maleimide decreases the –SH uptake as shown by citraconimide.

The mitotic inhibition of the imides exhibits a picture which is very different from the mitotic inhibition observed in the maleic acid group. Maleimide is less active than maleic acid, whereas citraconimide is quite active, although the free acid was inactive. The readiness of the maleimides to add sulphydryl compounds and the presence of glutathione in the medium used for the tissue cultures (Berger and Peters, 1933) may show the way for a reasonable explanation of these results. Experiments in this direction are in progress.

Summary

1. The antimitotic activity of maleimide (I), citraconimide (II), N-ethylmaleimide (III), and of succinimide (IV) has been tested in tissue cultures of chick fibroblast. The unsaturated imides (I, II, III) were active in the concentration range of $10^{-4}M$. The highest activity was shown by III, the methyl derivative of maleimide. The saturated imide (IV) was inactive.

2. The –SH uptake of the unsaturated imides has been determined. The introduction of a methyl group in maleimide decreases its reactivity towards –SH compounds.

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"CONSTANT FLOW" ORGAN-BATH TECHNIQUES

BY

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It has remained the common practice, since Magnus (1904) first showed that an excised strip of intestine would continue its rhythmic contractions if suspended in warm oxygenated Ringer-Locke solution, to change the contents of an organ bath by a *series* of washings. A drug is injected to give a known final concentration, rapid mixing being brought about by the stream of oxygen bubbles passing up through the bath; then, when the response of the muscle has been recorded, the solution is flushed out and replaced with fresh Ringer-Locke solution. Good techniques of this kind have been described recently by Chen, Ensor, and Clarke (1948) and by Miller, Becker, and Tainter (1948).

That it might be advantageous for some purposes to change the contents of an organ bath gradually instead of intermittently occurred to us in the course of experiments in which blood vessels were perfused at a constant rate. These studies (Fastier, 1948; Fastier and Reid, 1948) suggested that the duration of pharmacological effects observed under such conditions might be no less significant than their initial magnitude; for when a common site of action is indicated, the more active of two stable drugs is likely to be the one whose structural features, apart from providing the necessary "active groups," permit stronger adsorption at the site of action (Albert, 1944; Pfeiffer, 1948). It seemed important to check the idea that at least some of the drugs which have lasting effects upon preparations of this type are ones which are retained tenaciously by the muscle in the face of constant washing with the perfusing salt solution. Accordingly we sought a technique which would enable the outflowing Ringer's solution to be collected for analysis at the same time as the pharmacological effects of an added drug were being recorded.

The first type of apparatus devised for the purpose utilized a constant output pump (Messrs. Palmers' model F31), and the most compact form is illustrated in Fig. 1B. Well-oxygenated Ringer's solution is pumped from the reservoir *R* into the thermostatically controlled vessel *V*, which in turn maintains a constant temperature in the small organ bath *B*. Ringer's solution is forced into the bath *B* at a constant rate via the rubber tubing *I*, which also provides a convenient site for the injection of drug solutions. We have found that even when the pendulum movement of a muscle strip is quite vigorous, reasonably constant changes in tonus can be obtained by injecting such drugs as adrenaline, histamine, and acetylcholine in fixed small doses (Fig. 2). A good base-line is provided meanwhile by the general level of tonus. The Ringer's solution finally escapes through the overflow *O*, where successive portions may be collected for analysis.

The arrangement shown in Fig. 1A does not require a mechanical pump, a steady flow of Ringer's solution

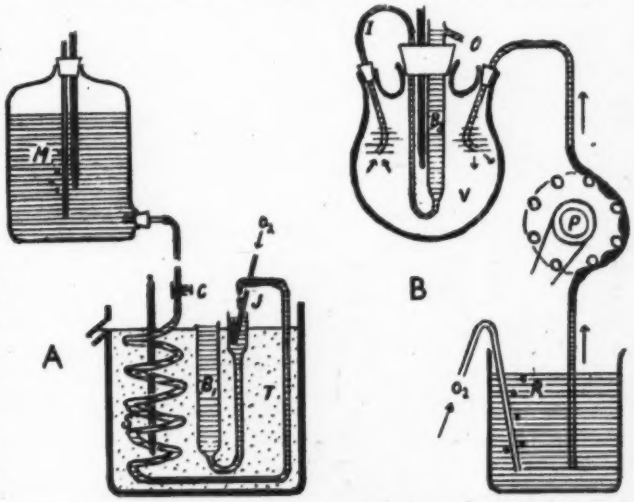

FIG. 1 (schematic).—Two types of apparatus permitting a slow, steady flow of Ringer's solution through organ baths (B_1 and B_2).

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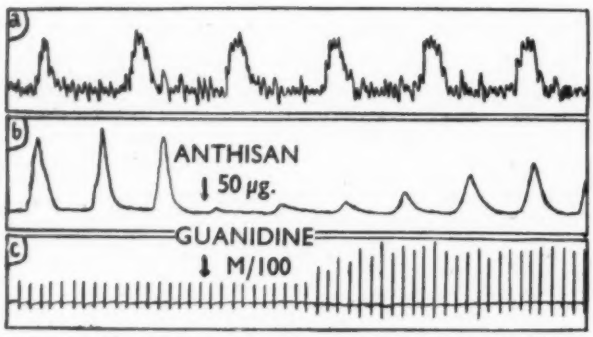

FIG. 2.—Facsimiles of kymograph records obtained by means of constant flow organ-bath techniques.

(*a*) Rabbit ileum washed with Ringer-Locke solution. The increases in tonus were produced with 0.2 μg. doses of acetylcholine injected at 3-min intervals. Pumping rate 30 c.c. per min.

(*b*) Guinea-pig ileum washed with Krebs-Henseleit solution. Histamine (5 μg.) injected at 4-min. intervals. "Anthisan" (50 μg.) injected at the arrow 1 min. before the fourth injection of histamine.

(*c*) Frog sartorius stimulated electrically at 10-sec. intervals. Guanidine hydrochloride (*M*/100) added to the perfusing Ringer-Harvey solution at the arrow. The effect of the stimulus is soon potentiated.

being secured by means of the Mariotte bottle *M*. The screw-clamp *C* adjusts the output. After passing through the spiral *S* in the thermostatic bath *T*, the Ringer's solution drips into the receiving chamber *J*, where it may be further oxygenated before it reaches the preparation. Drugs are injected here. In order to decide upon the best shape for the bath it has been found helpful to watch how a small quantity of injected dyestuff streams past the preparation ; this shows whether a drug solution will be distributed satisfactorily in transit.

To what extent the various principles followed —constant flow of Ringer's solution, "external" oxygenation and addition of drugs, the strip filling up most of the bath and always immersed in Ringer's solution—have been used previously, has proved almost impossible to ascertain. This is due not only to the widespread use of organ-bath technique but also to the relative infrequency with which it has been described on its own account. In a paper on the assay of substances liberated from adrenergic nerves, Gaddum, Jang, and Kwiatkowski (1939) have shown that it is very convenient to drip the perfusate from the organ under investigation on to a strip of hen's rectal caecum. Later Kwiatkowski (1941) successfully modified this dripping technique for the assay of histamine. More recently Schild (1947) has wellnigh perfected a method based upon the removal by suction of the excess of Ringer's solution entering a bath from a reservoir ; he has also demonstrated the advantages of adding an antagonist drug to the bath fluid before it reaches the gut.

SUMMARY

It has been found advantageous for some purposes to change the contents of an organ bath gradually instead of intermittently. Two types of apparatus based upon this principle are described.

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